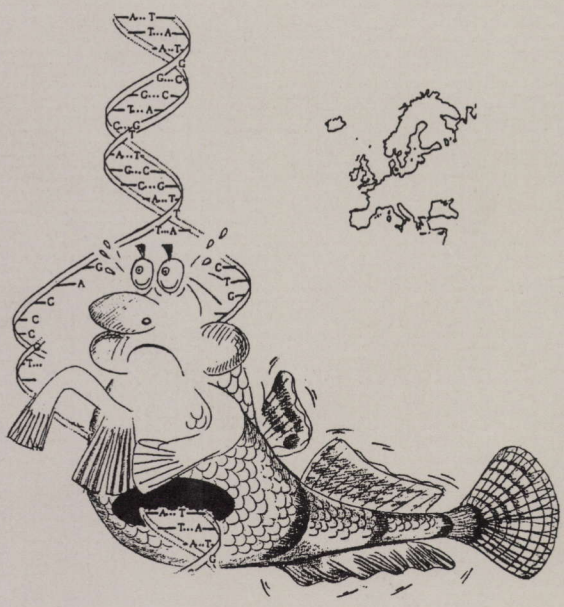
GENETIC STRUCTURE AND EVOLUTIONARY PATTERNS OF *POMATOSCHISTUS* GOBIES ON THE EUROPEAN CONTINENTAL SHELF AND IN THE MEDITERRANEAN SEA

Els Gysels



PhD THESIS

KATHOLIEKE UNIVERSITEIT LEUVEN
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DEPARTEMENT BIOLOGIE
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Genetic structure and evolutionary patterns in *Pomatoschistus* gobies on the European Continental Shelf and in the Mediterranean Sea

Genetische structuur en evolutieve patronen van *Pomatoschistus* grondels op het Europees Continentaal Plat en in de Middellandse Zee

Promotor: Prof. Dr. F.A.M. Volckaert

Proefschrift voorgedragen tot het behalen van de graad van Doctor in de Wetenschappen door:
Els Gysels

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Paper V (Pampoulie *et al*, submitted manuscript) has been included in this thesis with the permission of the first author C. Pampoulie, who was responsible for the microsatellite analysis of the sand goby on the Belgian Continental Shelf and the southwestern coast of The Netherlands, (1) because of its relevance to the interpretation of small-scale genetic structure in *Pomatoschistus* sp., and (2) in order to assess the performance of distinct genetic markers for disclosing population structure in sand gobies.

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LIST OF PAPERS

Allozyme variation and genetic divergence in the sand goby, *Pomatoschistus minutus* (Teleostei: Gobiidae)

A modified version of this paper has been accepted for publication in the Journal of the Marine Biological Association of the UK as a short paper with the following authors: Stefanni S, Gysels ES, Volckaert F and Miller.

ABSTRACT

Samples of the widely distributed sand goby *Pomatoschistus minutus* have been investigated genetically from seven localities in the North-eastern Atlantic Ocean, the North Sea, the western Mediterranean Sea and the Adriatic Sea. Levels of genetic diversity and differentiation were assessed with cellulose acetate (CAGE) gel electrophoresis for nine enzyme systems. Pair-wise F_{ST} values between samples point to a reduction or even absence of gene flow between the Adriatic and the other samples, including the western Mediterranean Sea (multi-locus F_{ST} = 0.321; multi-locus G_{ST} = 0.287). The differentiation between the Adriatic sample and all the others was of the same order of magnitude as between *P. minutus* and its closest relative, *P. lozanoi.* The sample from the Adriatic Sea was differentiated from the other samples mainly by allele frequency differences at the lactate dehydrogenase loci *LDH-A** and *LDH-C** (CAGE). At the sampling sites outside the Adriatic Sea the sand goby shows the typical features of a marine fish with a high level of gene flow and a low degree of genetic differentiation, which is roughly ten times lower than when the Adriatic *P. minutus* are included (multi-locus F_{ST} = 0.019; multi-locus G_{ST} = 0.028).

PAPER II

Recurrent and historic gene flow of the sand goby *Pomatoschistus minutus* on the European Continental Shelf and in the Mediterranean Sea

Gysels ES, Hellemans B, Patarnello T , Volckaert FAM

ABSTRACT

Phylogeographic patterns of the sand goby, *Pomatoschistus minutus* (Gobiidae, Teleostei) were studied by means of sequence and Single Stranded Conformational Polymorphism analysis of a 283 bp fragment of the cytochrome b locus in the mtDNA. A total of 228 individuals sampled at thirteen sites throughout the species' distributional range revealed a moderate level of diversity and a low, but significant level of overall genetic differentiation. The highest amount of population subdivision was detected between the samples from the northeastern Atlantic Ocean and the Mediterranean Sea, which was mainly due to differentiation between the *P. minutus* from the Adriatic Sea and all the other sand gobies, including the samples from the western Mediterranean Sea. Limited genetic differentiation with a weak pattern of isolation-by-distance was recorded in the western Mediterranean Sea, the Atlantic Ocean and the Baltic Sea. Phylogeographic analysis suggested a contiguous range expansion in the Atlantic and Baltic basins and evidence for a glacial refugium in the southern North Sea. Baltic samples showed evidence for lineage sorting by the presence of the most common Atlantic haplotype, with source genotypes from the North Sea. In the sand gobies from the western Mediterranean Sea a high number of endemic haplotypes as well as the most common Atlantic haplotype were recorded in appreciable frequencies. We suggest that this might be explained by a secondary contact between different mitochondrial lineages, which evolved in allopatry.

PAPER III

Phylogeographic patterns of the common goby, *Pomatoschistus microps*, a small demersal fish along the northeastern Atlantic coasts and in the Mediterranean Sea

Gysels ES, Hellemans B, Pampoulie C, Volckaert FAM

ABSTRACT

The phylogeographic patterns of a small marine fish, the common goby, *Pomatoschistus microps* (Krøyer 1838), were assessed along the northeastern Atlantic coasts. A combination of two genetic markers was employed: cellulose acetate allozyme electrophoresis (CAGE) and sequence analysis of a 289 bp fragment of the mitochondrial locus cytochrome b. Twelve coastal sites were sampled,

ranging from central Norway and the British Isles to Portugal and the Western Mediterranean Sea. Both markers were congruent in revealing significant differences between most populations and a pattern of isolation-by-distance along the European coasts. Phylogeographic analyses yielded a shallow branching structure with four groups; three were confined to the Atlantic basin and showed a starlike pattern, which may be due to either a relatively recent (postglacial) population expansion after a bottleneck or a founder event. The fourth group contained a central haplotype occurring at the edges of the species´ distribution (resp. the Norwegian and Mediterranean Sea) accompanied by a few rarer variants, which were restricted to the Mediterranean Sea. A genetic break was observed around the British Isles, with distinct haplotypes dominating at either side of the English Channel. Gene flow analysis suggested that recolonisation of the North Sea and the coasts of western Scotland and Ireland may have taken place from a glacial refugium the Southern Bight of the North Sea. Alternatively, the present distribution of the haplotypes, which corresponds to a Lusitanian/Boreal distribution, may be influenced by temperature-dependent selection. A significantly negative correlation between the degree of genetic diversity and latitude was recorded both for mtDNA and allozymes in the Atlantic basin. Historical factors such as founder effects during recolonisation of formerly glaciated areas are probably responsible for the loss of genetic variation at higher latitudes in the northeastern Atlantic Ocean.

PAPER IV

Small-scale genetic variation in relation to environmental heterogeneity in the marine gobies *Pomatoschistus minutus and P. lozanoi* (Teleostei)

Gysels ES, Leentjes V, Volckaert FAM

ABSTRACT

Genetic variation was assessed at 14 allozyme loci in estuarine, coastal and offshore samples of lozano´s goby, *Pomatoschistus lozanoi* and the sand goby, *P. minutus*. Samples were taken in habitats on the Belgian Continental Shelf and in the Schelde estuary subject to various degrees of environmental heterogeneity in order to assess (1) whether any correlation could be found between environmental heterogeneity and genetic diversity and (2) if so, if this could be linked to fitness parameters. No significant differences in levels of genetic diversity were recorded between

estuarine, coastal and offshore samples in either species. Homozygotes showed a slightly lower condition factor than heterozygotes in both species but the difference was not significant. A temporally stable gradient in allele frequencies at the two-allele locus *GPI-A** was observed in *P. lozanoi,* differentiating the samples in an estuarine, coastal and offshore group. We suggest that these differences might be maintained by balancing selection at locus *GPI-A**.

PAPER V

Evidence for fine scale genetic structure and estuarine colonisation in a high gene flow marine goby (*Pomatoschistus minutus*)

Pampoulie C, Gysels ES, Maes GE, Hellemans B, Leentjes V, Jones AG, Volckaert FAM

ABSTRACT

Marine fish seem to experience forces where homogenisation of populations should be the rule; yet evidence for structure, even on a small scale, is growing. We assessed genetic diversity and differentiation in 15 samples of the sand goby *Pomatoschistus minutus*, (Pallas 1770) (Gobiidae, Teleostei) from four major environments within the Southern Bight of the North Sea, using seven microsatellite and thirteen allozyme loci. Despite the high dispersal potential of the sand goby, microsatellite loci revealed a moderate level of differentiation (overall F_{ST} = 0.026; overall R_{ST} = 0.058). Both an hierarchical analysis of molecular variance and multivariate analysis revealed a significant differentiation ($P < 0.01$) between estuarine, coastal and marine samples with microsatellites but not with allozymes. Comparison among the different estimators of differentiation (F_{ST} and R_{ST}) pointed to possible historical events and contemporary habitat fragmentation. We conclude that this genetic structure was mainly due to the colonisation of the estuary during its establishment in the early Holocene, and to restricted actual gene flow. All samples can be assigned to two breeding units localised in estuarine and coastal region. Despite the distinction, there were indications of a complex and dynamic spatio-temporal structure, which is most likely, determined by the local current regime and the behavioural ecology of sand gobies.

CHAPTER I

INTRODUCTION AND GENERAL SCOPE OF THE THESIS

In this chapter we present an overview of the principal mechanisms responsible for population genetic structure in marine organisms and the methods used for studying population differentiation. We discuss this in relation to the three species of marine fish under study: the sand goby, *Pomatoschistus minutus* (Pallas, 1770), Lozano´s goby, *Pomatoschistus lozanoi* (de Buen, 1923), and the common goby, *Pomatoschistus microps* (Krøyer, 1838) (Teleostei, Gobiidae). We summarise the available data on (1) the biology of these species and (2) past and present-day oceanographic features throughout their distributional range in order to formulate hypotheses concerning their genetic structure.

1. Genetic structure and gene flow of marine organisms

The genetic structure of marine organisms is determined by several factors: (1) present-day physical oceanographic features, enhancing either retention or dispersal of the pelagic eggs or larvae (Lundy *et al*, 1999; Suneetha & Salvanes, 2001), (2) historical (vicariance) events (Nesbø *et al*, 1999; Planes & Fauvelot, 2002), (3) behaviour (Ruzzante *et al*, 1998; Beheregaray & Sunnucks, 2001) and (4) selective pressure from the environment (DiMichele *et al*, 1991; Lemaire *et al*, 2000).

Genetic studies point indeed to high migration rates and low levels of intraspecific differentiation in marine species, especially in comparison with terrestrial and freshwater organisms (for a review, see Ward *et al*, 1994). The duration of the larval stage is negatively correlated to the amount of population differentiation (Doherty *et al*, 1985; Waples, 1987). On the other hand, estimates of passive dispersal by oceanic currents might be grossly overestimated (Cowen *et al*, 2000). Fronts, current patterns such as gyres and eddies, bottom topography and climatic barriers may act as retention mechanisms for the migration of pelagic larvae and adults (Dickey-Collas *et al*, 1997; Lessios *et al*, 1999). Sinclair (1988) argues in his 'member-vagrant' hypothesis for highly vagrant fish species that local currents function as a mechanism for retention of the fittest 'members' of the local community. The less fit 'vagrants' show a higher rate of dispersal but risk not to find a suitable habitat or partner, thus representing a loss to the gene pool. Hence, a certain degree of philopatry is required and members should show homing behaviour towards the spawning

area. Alternatively, McQuinn (1997) suggests that the metapopulation concept might be better suited to explain intraspecific differences in marine populations. The metapopulation concept assumes a large number of sites each supporting a single local population, which has a certain probability of extinction. Vacant sites are recolonised by migrants from within the metapopulation. Genetic surveys on highly vagrant marine species yield evidence for a subtle genetic structure using high-resolution DNA markers (Stepien, 1999; Bahri-Sfar *et al*, 2000), which seems to confirm these assumptions. On the other hand, both larvae and adult fish of several marine species are known to make use of selective tidal transport for migration (Metcalfe *et al*, 1990; Arnold *et al*, 1994) and thus may actually travel further than expected by purely passive dispersal.

Another major factor in shaping the structure of a species is its evolutionary history (Bernatchez & Wilson, 1998; Hundertmark *et al*, 2002). Species boundaries have shifted back and forth in function of vicariance events and climatic change, including glaciation. These have had a profound effect on the levels of genetic variation and population differentiation (Magoulas *et al*, 1996; Benzie & Williams, 1997). Phylogeographic analyses of several species have yielded evidence for periodic population extinction and recolonisation, linked to Pleistocene glaciations (Grant & Bowen, 1998). During the Pleistocene glaciations species have been pushed back southward along the Atlantic coasts and subsequently recolonised the coastal areas of North-western Europe following the glacier's retreat. In Europe, the coasts of the Iberian Peninsula, the North Sea and certain ice lakes east of the Baltic Sea are considered glacial refugia for aquatic species (Osinov & Bernatchez, 1996; Koljonen *et al*, 1999; Consuegra *et al*, 2002).

2. Genetic methods used for assessing population structure

Early pioneers such as Wahlund (1928) and Wright (1943) established the theoretical principles of population genetics long before any practical attempt was made at disclosing genetic structure in living organisms. The first population genetic surveys date back to the 1960's with the use of haemoglobine for assessing population structure in marine fish such as gadoids (Sick, 1961; Møller, 1966; Jamieson, 1967). The discovery of a large amount of allelic variation at protein loci prompted the development of allozyme electrophoresis as a widely used tool in the study of genetic structure within and between species (See Avise, 1975, for a review). Allozyme electrophoresis is a relatively easy technique and large amounts of samples can be processed in a short period. However, the use

of proteins had serious shortcomings. Only a small proportion of the genomic variation is revealed. Not all mutations are translated into different alleles, and different alleles may show an identical mobility on a gel (Utter *et al*, 1987). Thus, a more subtle population structure is likely to go unnoticed. Moreover, the debate between 'neutralists' and 'selectionists' questioned the usefulness of these markers. The high level of polymorphism found at protein loci seemed to support the view of Kimura (1979), who stated that most of the variation at a locus is selectively neutral. At the other hand, many studies showed clear evidence for selective pressure in maintaining allele frequencies (Christiansen & Frydenberg, 1974; Mork *et al*, 1983; Ben-Shlomo & Nevo, 1988; Lemaire *et al*, 2000). If alleles at certain loci perform better than others in a given environment (e.g. temperature, pollution, salinity), then frequencies at these loci will be determined by natural selection rather than genetic drift. A growing number of studies prove selective mortality of certain genotypes and local adaptation to environmental variables such as temperature, salinity or pollution (see Mitton, 1997 and Eanes, 2002, for a review). Clinal variation in allele frequencies in function of environmental variables has been reported on a scale of meters (Johannesson *et al*, 1995) to hundreds of kilometers (Oakeshott *et al*, 1982; Maes & Volckaert, 2002). Yet, in spite of the large amount of circumstantial evidence, it remains very difficult to actually prove selection acting on a locus, as this requires detailed biochemical experiments (but see Watt, 1983; Watt *et al*, 1983; Zera, 1987; DiMichele *et al*, 1991). Nevertheless, allozymes remain useful markers for detecting population structure and resolving taxonomic relationships between species; they are still widely used as a genetic marker (e.g. Mattiangeli *et al*, 2000; Suneetha & Salvanes, 2001; Pujolar *et al*, 2002).

The advent of modern DNA techniques with the invention of the Polymerase Chain Reaction technique (PCR) and the development of universal primers (e.g. Kocher *et al*, 1989; Cronin *et al*, 1992) gave a new impetus to population genetics. For the first time a large amount of DNA could be amplified from a minute tissue sample and a much higher resolution could be achieved. Indeed, several DNA studies have yielded evidence for population structuring whereas allozymes did not (Pogson *et al*, 1995; Piel & Nutt, 2000). Especially the mitochondrial DNA became popular as a marker because of its smaller effective population size and faster rate of evolution (Park & Moran, 1994). Mitochondrial DNA is transferred almost exclusively maternally and no recombination takes place. Thus, this marker is well suited for genealogical studies, leading to the development of phylogeography. Phylogeography deals with the processes determining the geographic distribution of genealogical lineages within and among species (Avise, 2000). As such,

this discipline combines biogeography and phylogenetics and tries to explain the structure and evolution of species and populations of species in terms of historical events.

Earlier mtDNA studies were mainly based on Restriction Fragment Polymorphism Analysis (RFLP) (e.g. Avise *et al*, 1987; Arnason *et al*, 1992; Cronin *et al*, 1992), which is an indirect method for assessing the genetic variation at a given locus. The development of automated sequencers allowed a quick sequencing of the nucleotides, thereby obtaining the highest possible resolution. Disadvantages of this method are associated with the high running costs and the sophisticated infrastructure required. An alternative for sequencing all individuals in a given sample is Single Stranded Conformational Polymorphism (SSCP), where mutational differences are translated into different mobilities on a gel (Orita *et al*, 1989). The various haplotypes distinguished by SSCP can subsequently be sequenced. Mitochondrial DNA was initially promoted as being less subjected to selective pressure than proteins, but this putative neutrality has been questioned as well (Ballard & Kreitman, 1995). Most genes in the mitochondrial DNA are coding and thus their evolution may also be subjected to selective pressure. Because mtDNA does not undergo recombination, it is actually behaving as a single locus, and thus selection acting on a gene will cause all others to be selected as well. For example, the low amount of mtDNA diversity in humans was originally considered the effect of a founder event (see Avise, 2000, for a review), but this may as well be the result of selection acting on certain loci (Mishmar *et al*, 2003).

In more recent years, the use of microsatellites in population genetics has been growing fast (reviewed in DeWoody & Avise, 2000). Microsatellites are non-coding parts in the nuclear DNA, which consist of repeats of several nucleotides. They evolve very quickly and are highly variable. Thus, microsatellites are considered very useful for assessing more recent processes of population divergence or disclosing the shallow but significant population structure, which is typical for many marine species with a high potential for dispersal. Several studies provide indeed evidence for genetic structure in migratory species where no differentiation was detected using mtDNA or allozymes (Ruzzante *et al*, 1998; Nesbø *et al*, 2000; Wirth & Bernatchez, 2001). However, caution is warranted when interpreting microsatellite data. The extremely high variability and the consistent heterozygote deficit, which are often associated with microsatellites, make interpretation difficult. Furthermore, recent studies suggest that these putative neutral (for non-coding) markers may not always behave neutrally either (Dufresne *et al*, 2002). Thus, for making reliable inferences about population structure and/or history, a combination of several markers, nuclear and mitochondrial and including as many loci is possible, is recommended (Allendorf & Seeb, 2000).

3. The genus *Pomatoschistus*

3.1. Distribution and ecology of *Pomatoschistus minutus* (Pallas, 1770), *P. lozanoi* (de Buen, 1923) and *P. microps* (Kroeyer, 1838)

Gobies (Teleostei, Gobiidae) are a speciose family of relatively small, demersal fish occurring mainly in tropical and subtropical coastal areas, although a considerable number of species occur in freshwater and brackish water as well (Miller, 1986). Members of the genus *Pomatoschistus*, which comprises eleven species, are very abundant along the Atlantic European coasts. Among these, the sand goby, *P. minutus* (Pallas, 1770), Lozano's goby, *P. lozanoi* (de Buen, 1923) and the common goby, *P. microps* (Krøyer, 1838), are among the most common fish species in the coastal areas of Atlantic Europe. They play an important role in marine coastal ecosystems as a predator of meiobenthos and plankton (Hamerlynck & Cattrijsse, 1994), as prey for a number of commercially important fish species such as cod and flatfish (Salvanes & Nordeide, 1993) and as a host for marine parasites (Zander *et al*, 2002). No commercial fisheries for these species exist, but they are often present as by-catch in shrimp fisheries, and may occur among the small fish offered at Mediterranean markets (Miller, 1986).

All species belonging to the genus *Pomatoschistus* display courtship behaviour whereby a male establishes a territory, builds a nest under an empty bivalve shell and courts a female (Fonds, 1973). The female inspects the nest and decides whether to deposit her eggs. These are attached at the ceiling of the nest and are subsequently fertilised by the male, who protects and fans them during several weeks until they hatch. Territorial males guard batches of eggs from several females, that deposit eggs in various nests. Sneaking behaviour, where a non-territorial male enters the nest of another male and tries to fertilise the eggs has been observed both for the sand goby and the common goby (Magnhagen, 1994; Forsgren, 1997) and seems to be a strategy used by smaller males (Magnhagen, 1998). Usually the nest holder expels the intruder but a fairly high rate of successful sneaking fertilisation has been observed. Up to one third of sand goby nests contained eggs fertilised by sneakers (Jones *et al*, 2001b). Upon hatching the larvae leave the nest and dwell in the plankton during one to two months (Healey, 1971). After metamorphosis they become demersal.

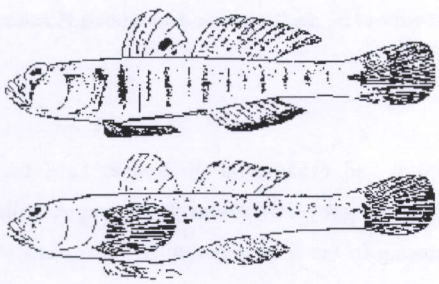
Of the three species being studied, the sand goby, *P. minutus,* has the largest area of distribution. It occurs along the Atlantic coasts of Europe from the north of Norway (Tromsø) and the Faroe Islands (Miller, 1986 and references herein) to the northern African coasts, in the Baltic

Sea, the Mediterranean Sea and the Black Sea. The common goby, *P. microps* (Krøyer, 1838) occurs from central Norway (Trondheimsfjorden) to the south of Portugal and Atlantic Morocco, around the British Isles and in the Baltic Sea. In the western Mediterranean Sea its distribution is limited to the Gulf of Lions and Corsica (Bouchereau & Guelorget, 1997). Lozano's goby, *P. lozanoi*, has the smallest range of distribution and can be found along the Atlantic coast of Denmark down to the south of Portugal. *P. lozanoi* has not been recorded from the Mediterranean Sea or the Baltic Sea (Miller, 1986). A schematic view of the morphological differences between the species is presented in Figure 1.1. Data on ecological and morphological differences between these three species have been compiled from literature and are summarised in Table 1.1.

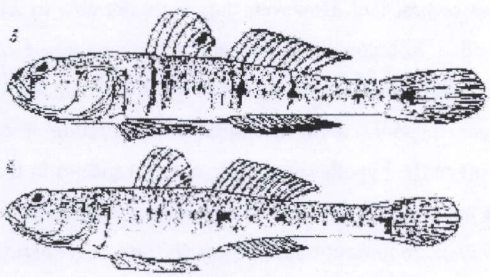
P. minutus and *P. lozanoi* are morphologically very similar and the latter was considered to be a subspecies until recently (*P. minutus minutus* and *P. minutus lozanoi* respectively). Based on distinct ecological, morphological and biochemical differences they have been assigned the status of distinct species (Fonds, 1973; Webb, 1980). The most reliable morphological criterion for distinguishing between these two species is the papilla pattern on the head (Miller, 1986) (Fig. 1.1), but differences in colour pattern have also been described (Webb, 1980; Hamerlynck, 1990). *P. minutus* grows larger than *P. lozanoi* (with a maximum size of 95 mm versus 80 mm respectively) and has a more sandy-grey colour while *P. lozanoi* is rather brownish. Juveniles are notoriously difficult to identify morphologically. Unambiguous biochemical identification is possible through diagnostic alleles at several allozyme loci (Webb, 1980; Wallis & Beardmore, 1984a), but this requires freshly frozen tissues.

P. minutus and *P. lozanoi* are grouped together in the '*P. minutus complex*' together with the closely related deep-water species *P. norvegicus* (Collett, 1902). Although *P. minutus, P. microps* and *P. lozanoi* occur sympatrically throughout a large part of their distributional range, they show a clear segregation in habitat, feeding and spawning niche. *P. minutus* is an estuarine-marine species and rather a generalist in trophic niche, feeding mainly on epibenthic and hyperbenthic prey. *P. lozanoi* is more of a specialist, preying on hyperbenthic animals in the water column (Hamerlynck *et al*, 1990). *P. lozanoi* has a more neritic distribution than *P. minutus* (Fonds, 1973), although large numbers are often recorded in estuaries (Claridge *et al*, 1985; Maes *et al*, 1998a). At times a considerable dietary overlap exists between the two species (Hamerlynck & Cattrijsse, 1994). *P. microps* has an estuarine-coastal distribution, adult specimens being rarely encountered in the offshore marine environment. The common goby spawns close to shore in water no deeper than 1 m and is capable of reproducing both at sea and in estuaries (Healey, 1972). *P. minutus* spawns in

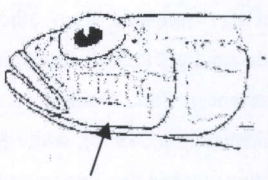
deeper water (up to 25 m) and usually at sea (Fonds, 1973), although spawning in estuarine or brackish-water areas has been reported as well (Vaas, 1970; Arruda *et al*, 1993). *P. lozanoi* apparently only reproduces in a marine environment at depths between 10 and 25 m (Fonds, 1973). *P. minutus* and *P. microps* often occur sympatrically in estuarine and shallow coastal areas. Interspecific competition may be reduced by depth segregation (Doornbos & Twisk, 1987; Maes *et al*, 1998a). *P. minutus* spawns in slightly deeper water than *P. microps* and chooses also larger shells for its nests than *P. microps* (Nellbring, 1993). Magnhagen & Wiederholm (1982) showed that the choice of food and habitat depended on whether *P. minutus* and *P. microps* occurred sympatrically or alone.



P. microps



P. minutus



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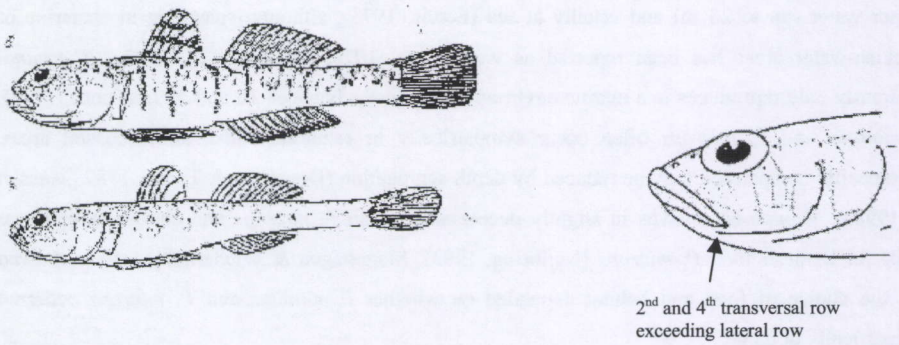

P. lozanoi

Fig.1.1: *Pomatoschistus minutus, P. lozanoi* and *P. microps*. Details of the papillae pattern distinguishing *P. minutus* from *P. lozanoi* are presented on the right (Miller, 1986).

Considerable within-species ecological, morphological and behavioural differences have been recorded for geographically distinct populations of the **sand goby, *P. minutus***. For example, Miller (1986) considers the population of *P. minutus* occurring in the Mediterranean Sea as a separate subspecies, *P. minutus elongatus*, based on morphological characteristics. Mediterranean *P. minutus* carry a dark chin spot in the females and breast pigmentation in both sexes. Wallis & Beardmore (1983) found distinct allozymatic differences between *P. minutus* from the Bristol Channel (North Sea) and the lagoon of Venice (Adriatic Sea) at several loci. However, they were not able to tell whether these differences are due to reproductive isolation or if they are the consequence of selective pressure in the specific environmental circumstances in the lagoon of Venice. Neither is it known if *P. minutus* from other Mediterranean locations show a similar differentiation from Atlantic sand gobies, which would render support to the hypothesis of a separate subspecies in the Mediterranean Sea. Mediterranean *P. minutus* are annual and occur in lagoon-like environments (Bouchereau *et al*, 1990), while Atlantic *P. minutus* are present both in estuarine and fully marine environments, and can live up to two years (Healey, 1972). Atlantic *P. minutus* disappear from the coast in the winter and it is assumed that they migrate into deeper (warmer) water, once the temperature decreases below 2-3° C (Fonds, 1973). Both Mediterranean and Atlantic *P. minutus* perform a spawning migration towards the sea. *P. minutus* from the Baltic Sea differ also

morphologically from the Atlantic populations (Aarnio & Bonsdorff, 1993), but no genetic information is available to quantify the extent of differentiation. Moreover, the Baltic coast harbours two morphologically distinct ecotypes, being linked to sandy and rocky habitats respectively. These seem to stay in their own habitat for breeding (Pampoulie, pers. comm.). It is currently unknown whether any gene flow exists between these two morphs.

Similar ecological, morphological and behavioural differences have been described among geographic populations of the **common goby, *P. microps***. Baltic *P. microps* differ morphologically as well as ecologically from the Atlantic *P. microps*. Hence, a separate race for the Baltic *P. microps* has been proposed (Lawacz, 1965). Al-Hassan *et al* (1987) observed allozymatic differences between populations of *P. microps* along the coastlines of the British Isles, but the authors were unable to discriminate between temperature-dependent selection and reproductive isolation for explaining the observed population structure. In the Mediterranean Sea *P. microps* is annual and sedentary (Boucherau & Guelorget, 1997) contrary to Atlantic *P. microps*. Mediterranean *P. minutus* spawn between March and September (Pampoulie, 2001). In the northeastern Atlantic Ocean the species disappears from the coast during the winter season where the water temperature falls below 3-5° C (Jones & Miller, 1966). Reproduction starts only when the water temperature exceeds 10°C and the breeding season lasts from April to September (Rogers, 1988). The maximum lifespan is about 20 months. Apart from the study of Al-Hassan *et al* (1987), few if any population genetic data on the common goby are available for assessing whether the observed inter-population differences are due to either phenotypic plasticity or genuine reproductive isolation.

Lozano´s goby, *P. lozanoi*, has been less studied, which may be due to its preference for off-shore habitats and smaller range of distribution. Fonds (1973) noticed a more limited temperature and salinity tolerance in *P. lozanoi* and suggested a more neritic life-style than *P. minutus*. Despite its occurrence in estuaries they do not seem to spawn there (Arruda *et al*, 1993). Few data on geographically distinct populations of Lozano´s goby are available and hence little is known about morphological, ecological or genetic differences between allopatric populations of this species.

Table 1.1. Main ecological differences between *Pomatoschistus minutus*, *P. lozanoi* and *P. microps*. Data have been compiled from Jones & Miller (1966), Healey (1972), Fonds (1973), Webb (1980), Miller, (1986), Hamerlynck (1990), Hamerlynck & Cattrijsse (1994), Bouchereau & Guelorget (1997), Pampoulie *et al* (1999) and Pampoulie *et al* (2000).

	P. minutus	***P. lozanoi***	***P. microps***
ECOLOGY			
Area of distribution	northeastern Atlantic coasts from Tromsø to southern Portugal, Baltic Sea, Mediterranean Sea, Black Sea	northeastern Atlantic coasts from Denmark to southern Portugal	northeastern Atlantic coasts from Trondheim to southern Portugal, Baltic Sea, Western Mediterranean Sea (Gulf of Lions and Corsica)
Food preference	Mainly epibenthic and benthic animals, also zooplankton and hyperbenthos	(hyper) benthic invertebrates, juvenile *P. minutus*	Benthic and epibenthic invertebrates (amhipods, mysids, shrimps)
Habitat	Open and vegetated habitats	Variable	Vegetated when in sympatry with *P. minutus*; open habitat if alone
Depth range	0-70 m	0-80 m	6-325 m
Migratory behaviour	- Mediterranean: inshore spawning migration - Atlantic: inshore spawning migration but breeds also in estuaries - Off-shore thermal migration when water temperature < 2°C and >19°C	- Spawns at sea, no breeding in estuaries recorded - Off-shore thermal migration when water temperature < 2°C	- Possible spawning migration of estuarine populations in Atlantic populations, breeds also in estuaries - Off-shore thermal migration when water temperature < 3-5°C Mediterranean populations: sedentary

Table 1.1 (continued)

	P. minutus	*P. lozanoi*	*P. microps*
REPRODUCTION			
Spawning period	Atlantic: February-June (depending on latitude) Mediterranean: December-April	May-August (Wadden Sea)	Atlantic: April-September, depending on latitude Mediterranean: February-June
Spawning site	Marine or estuarine environment, from 0-25 m	Marine environment, 10-25m	Shallow marine or estuarine environment, max 1m depth
Fecundity	2000-4000 eggs per batch	2000-4000 eggs per batch	1000-3000 eggs per batch
Temperature for breeding	8-14 °C	8-14°C	10°C
Duration of pelagic larval stage	At least 1 month	At least 1 month	Six-nine weeks
KARYOLOGY			
Number of chromosomes	46	37	32

3.2. Previous genetic studies on *Pomatoschistus* sp.

Considering their abundance, ecological importance and peculiar reproductive behaviour, it is not surprising that *Pomatoschistus* sp. have been the subject of a large number of ecological and behavioural studies. However, genetic data of these species are scarce and have mainly focused on resolving the taxonomic status and the phylogenetic relationships within this genus. So far, most allozyme-based studies have been conducted by Webb (1980), Wallis & Beardmore (1980, 1983, 1984a,b), Miller *et al* (1993), Sorice & Caputo (1999) and Stefanni (1996, 2000a). The screening of allele frequencies at 31 loci within seven species belonging to the genus *Pomatoschistus* (Wallis & Beardmore, 1984a) confirmed most of the morphology-based systematic relationships. Phylogenetic studies of the Atlanto-Mediterranean gobies based on sequencing of the mtDNA and nDNA have been conducted by Penzo *et al* (1998) and Huyse (2002). Microsatellites have been employed as

well, but mainly for studying sneaking behaviour and sexual selection in *P. minutus* (Jones *et al*, 2001 a,b; Malavasi *et al*, 2001).

Webb (1980) confirmed the species status of *P. minutus* and *P. lozanoi* as opposed to earlier taxonomy, where *P. lozanoi* was considered a subspecies of *P. minutus* (respectively *P. minutus lozanoi* and *P. minutus minutus*). This taxonomic revision was already proposed by Fonds (1973), based on ecological differences between these putative subspecies in the Wadden Sea. The striking differences between *P. minutus* and *P. lozanoi* at several allozyme loci *(GPI-A*, GPI- B*, CK-A**) and haemoglobins, as well as differences in number of chromosomes recorded by Webb (1980), confirmed that they should be considered as genuine species instead. *P. minutus* is monomorphic for a slower moving allele than *P. lozanoi* at locus *CK-A**. Both species segregate also at the *GPI* loci but alleles are not completely fixed. The surveys of Wallis & Beardmore (1980, 1984a) revealed that both species possess also distinct alleles at the loci *PEP-1** and *ALAT-1 **.

P. minutus and *P. lozanoi* hybridise, though; Wallis & Beardmore (1980) claim even to have found evidence for an F2 hybrid. However, this was based on the genotype frequencies of only one individual out of more than 1700 fish screened at one locus (*PEP-1**) and did not involve any crossing experiments. F1-hybrids are clearly distinguished from genuine *P. minutus* and *P. lozanoi* by the possession of two bands at the monomeric locus *CK-A** (Webb, 1980). This suggests that *P. minutus* and *P. lozanoi* are very closely related and must have speciated only recently; Wallis & Beardmore (1984a) suggest a speciation event about 2.5 Ma BP, based on allozymes. Huyse (2002) on the other hand, proposes a more recent speciation of about 1.73 to 1.13 Ma BP for speciation within the *P. minutus* complex, based 12S and 16S mtDNA.

Based on the screening of 31 allozyme loci, Wallis & Beardmore (1984b) found a correlation between the level of genetic variation within seven species of *Pomatoschistus* sp. and the degree of habitat heterogeneity in which the distinct species preferentially occur. However, Sorice & Caputo (1999) failed to find such correlation when assessing levels of genetic diversity at 20 allozyme loci in seven Mediterranean goby species. Likewise, Miller *et al* (1993) could not find any significant correlation between genetic variation and environmental heterogeneity in a survey of ten Mediterranean goby species, which were screened at 18 loci.

Few genetic studies deal with geographically distinct populations of *Pomatoschistus* sp. Wallis & Beardmore (1983) recorded nearly fixed differences at several allozyme loci (*LDH-A**, *LDH-C**) between *P. minutus* from Bristol Channel and the Lagoon of Venice (northern Adriatic Sea), and even postulated the existence of a fourth locus for the LDH enzyme in the Venetian

population, *LDH-0**, which would be completely unique in this species. Stefanni *et al* (1996) confirmed the differentiation at locus *LDH-A** but found no evidence for this putative fourth locus. Sequence analysis of the complete D-loop showed also differences between Adriatic and other sand gobies (Stefanni, 2000a). Huyse (2002) recorded significant differences at the 12S and 16S loci of the mtDNA between sand gobies from the Lagoon of Venice and the North Sea, suggesting the presence of a distinct species of the sand goby in the northern Adriatic Sea.

Huyse (2002) reported no significant differences at the mitochondrial loci 12S and 16S between specimens of *P. minutus* from the North Sea and the western Mediterranean Sea, but in this study only 3 (North Sea) and 2 (western Mediterranean Sea) individuals were studied, primarily for resolving phylogenetic relationships among species. Moreover, a slower evolving marker was used, which may not be appropriate for resolving recent processes of population divergence. Al-Hassan *et al* (1987) found significant differences in allele frequencies at the *GPI** loci for the common goby, *P. microps,* collected at 20 sites around the British Isles. It was not clear, however, if this had to be explained as some form of temperature-dependent selection on the respective alleles, or else allopatric divergence during postglacial recolonisation of the British Isles by the common goby.

Thus, in this study we aim to fill the gap in the current knowledge of the genetic structure in geographically distinct populations of these widely spread species. More specifically, we will address following issues:

(1) assessing the taxonomic status of the northern Adriatic *P. minutus* with a combination of two independent markers (allozymes and mtDNA).

(2) Comparison of samples of *P. minutus* from the western Mediterranean with those from the Atlantic Ocean to check whether there exists a genetic basis for assigning subspecies status to the western Mediterranean sand gobies.

(3) Screening of the geographically distinct samples of *P. minutus* and *P. microps* for estimating their levels of genetic divergence throughout the distributional range.

3.3. Dispersal of Pomatoschistus sp.

3.3.1. Historical events influencing dispersal in Pomatoschistus sp.

The contemporary distribution of *Pomatoschistus* sp. along the European Atlantic coasts is the result of a postglacial northward range expansion of its distributional range after deglaciation at the end of the Pleistocene. The Pleistocene epoch is characterised by a series of glacial events followed by interglacials. During glaciation sea levels were much lower than today and an icecap covered large parts of northwestern Europe. A connection existed between Britain and the continent. Parts of the Southern North Sea were dry land and the English Channel served as a drainage system for rivers such as Schelde, Thames and Rhine (Gibbard, 1988). During the Last Glacial Maximum (22-18 ka BP), the ice cap extended as far south as southern Britain (Lowe & Walker, 1997). *Pomatoschistus* sp. require shallow water at a temperature of about 8-14 °C for breeding, and thus the ice-covered areas in the north were unsuitable habitats. Hence, during glaciation, *Pomatoschistus* sp. must have survived south of the ice-sheets, possibly along the Iberian or the northern African coasts, and expanded its range northward after the retreat of the sea ice. One of the possible consequences of such a putative range expansion should be the loss of genetic diversity in populations at higher latitudes (Hewitt, 1996) due to a rapid expansion into formerly glaciated habitats from population surviving in areas to the south. Alternatively, isolated populations of the species may have survived in glacial refugia in more northern parts. For example, a glacial refugium for trout has been proposed along the southwestern coast of Britain, near the outflow of the great rivers through the then-dry Strait of Dover (Hamilton *et al*, 1989; García-Marin *et al*, 1999). A glacial lake in the southern North Sea has also been proposed as a refugium for Atlantic salmon (Verspoor *et al*, 1999). Populations of *P. minutus* and *P. microps*, being able to live in brackish-water conditions, may have survived in such a glacial refugium along the margins of the ice sheets or in this glacial lake, and carried out a northward and/or westward recolonisation from their putative refugium. If so, the English Channel and the Southern North Sea could be a zone of secondary contact between migrants from the south expanding their range northward and resident populations.

Evidence for glacial refugia in lakes east of the Baltic Sea has been found as well. The occurrence of phylogeographically distinct lineages of Baltic salmon and trout suggests that recolonisation of the Baltic Sea has taken place both from refugia east of the Baltic Sea and from

the adjacent North Sea (Osinov & Bernatchez, 1996; Koljonen *et al*, 1999). Whether this hypothesis applies to *Pomatoschistus sp.* as well, or alternatively, that the North Sea was the only source of colonisation, may be inferred from a comparison of Atlantic and Baltic haplotypes.

The Mediterranean Sea served also as a refugium for many species. Although the impact of the Pleistocene glaciations on the Mediterranean Sea was not as dramatic as in the northeastern Atlantic ocean, the sea-level was lower during glacial periods. This resulted in the isolation of various basins, which were also isolated from the main Atlantic basin. Thus, marine Mediterranean populations might also have been isolated for a long time, from populations in adjacent bodies of water and from their Atlantic conspecifics, which eventually leads to allopatric speciation. For example, it has been suggested that Mediterranean blennies originated in isolation in Pleistocene refugia within the Mediterranean Sea (Geertjes *et al*, 2001).

3.3.2. Influence of current oceanographic patterns on large-scale dispersal of Pomatoschistus sp.

Pomatoschistus sp. are considered poor swimmers as the pelvic fins of adults are fused into some kind of suction disc, allowing them to keep their position in the current (Miller, 1986). Thus, large-scale dispersal and gene flow between populations occurs most likely at the pelagic larval stage. This extended pelagic larval phase provides a high potential for dispersal by oceanic currents; a substantial gene flow between geographic localities is not unlikely. However, as described above, adult *Pomatoschistus* sp. carry out spawning and thermal migrations. Although it is not known how far they migrate, selective use of tidal streams and oceanographic currents may enable them to cover large distances as well.

The extended distributional range of *P. minutus* and *P. microps* and the prolonged pelagic larval stage of these species provide an excellent opportunity to assess the importance of hydrodynamic forces on their dispersal along the Northeastern Atlantic coasts. Moreover, considering the similar mode of reproduction of these ecologically different species and hence a similar potential of dispersal, a comparison of the genetic structure between these two species will enable us to assess the importance of ecological and/or behavioural constraints on the actual dispersal pattern.

The northeastern Atlantic Ocean

Any passive large-scale larval dispersal of fishes including *Pomatoschistus sp.* gobies in the Atlantic basin is influenced by oceanic currents, the most important of these being the North Atlantic Current (NAC) and the Shelf Edge Current (SEC) (Fig.1.2). The North Atlantic Current is the extension of the Gulf Stream, branching northward and southward upon arriving at the edge of the European Continental Shelf and carrying warm water towards the European coasts. The Shelf Edge Current flows northward along the edges of the Continental Shelf from north-western Africa via Portugal, the Bay of Biscay and the west of the British Isles towards the Norwegian Sea. The merger of SEC and NAC results in a net northbound current along the western Irish and Scottish coasts towards the Norwegian trench (Norwegian Trench Current) and northward along the Norwegian coast (Norwegian Coastal Current), while an arm branches off both east and west off the Shetland Islands to flow southward along the eastern English coast and eastward towards the Skagerrak as the Fair Isles Current (FIC) and the East Shetland Atlantic Inflow (ESAI) (Turrell, 1992) (Fig.1.3). The FIC and EASI provide the principal input of Atlantic water and nutrients into the northwestern North Sea. Atlantic water enters the North Sea via the English Channel, resulting in a northward flow through the English Channel into the southern North Sea (Southern Bight). An additional branch of Atlantic water flows northward through the deep, vertically stratified Celtic Sea and into the shallower, well-mixed Irish Sea. Between these adjacent seas a tidal mixing front, the Celtic Sea Font is generated during summer. The eastern Irish Sea is dominated by strong tidal currents and is consequently wellmixed throughout the year. In the western Irish Sea at the other hand, tidal currents are weak and an extensive gyre is formed there during spring and summer (Horsburgh *et al*, 2000), probably acting as a retention mechanism for pelagic larvae of fish and invertebrates (Hill *et al*, 1996; Dickey-Collas *et al*, 1997).

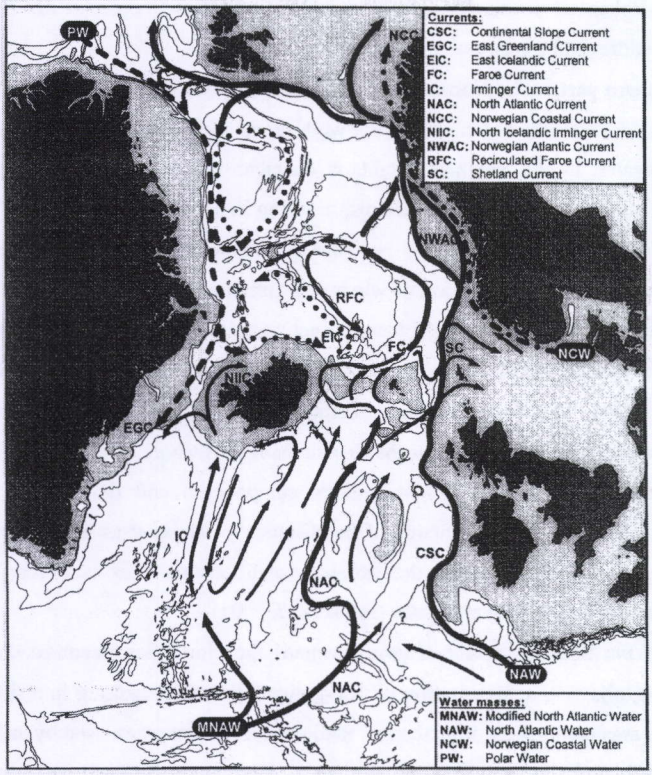

Figure 1.2: Current patterns in the Northeastern Atlantic (Hansen & Østerhus, 2000).

The North Sea

The North Sea comprises the English Channel, the Central North Sea, the Skagerrak and Kattegat and extends as far north as 62° N (Norwegian Trench). It receives both saline Atlantic water and brackish water from the Baltic Sea, as well as the outflow of major rivers such as the Rhine, the Elbe and the Thames (Ducrotoy *et al*, 2000). Most areas in the North Sea are vertically well mixed during the winter months, but stratify thermally in summer, separating shallow zones with strong tidal currents from deeper, stratified zones with weak tidal currents. Thermally stratified and mixed areas are separated by fronts, which are characterised by sudden changes in temperature and/or

salinity and an enhanced production of phytoplankton and fish (Young *et al*, 2002). They are associated with eddies and gyres, acting as larval retention zones.

The southern part of the North Sea (Southern Bight) is a shallow sandy area, continuously swept by strong tidal currents and consequently well-mixed all year round. The Belgian Continental Shelf (BCP), located in the Southern Bight, is characterised by the occurrence of sandbanks alternating with gullies and strong tidal currents, resulting in habitat patchiness and a high degree of heterogeneity. These sandbanks provide unstable environments, which are subject to constant changes in topography due to the action of winds and currents (Vincent *et al*, 1998; van der Molen & de Swart, 2001a) A gradient in abiotic factors, such as salinity, nutrient concentrations, sediment composition and turbidity, exists as a function of the distance to the coast and the Schelde estuary (Swertz *et al*, 1999). The Schelde estuary comprises a very heterogeneous environment that influences the coastal area as far away as the Zeelandbanken (Dubois, pers. comm.). Consequently, a number of biotic factors such as the species composition and diversity of the plankton, hyperbenthos and benthos vary in function of the distance to the coast as well (Vanaverbeke *et al*, 2000; Dewicke *et al*, 2003). Despite the variable conditions the area is a very important and productive habitat for fish and invertebrates (Beyst *et al*, 2001).

North of the Southern Bight (Oyster Ground) the North Sea becomes deeper and tidal currents decrease, allowing sedimentation of fine-grained material, resulting in a silty bottom. The boundary of this area comprises a frontal zone, the Frisian Front, characterised by an enhanced level of biodiversity (Dewicke *et al*, 2002) and serving as a boundary between southern and northern benthic communities.

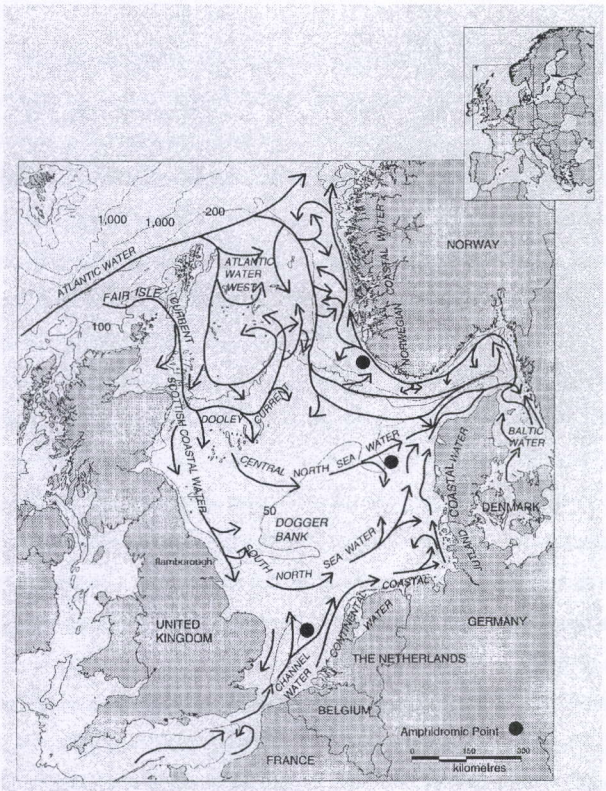

Figure 1.3: Predominant currents in the North Sea (After Turrell, 1992 and Ducrotoy *et al*, 2000).

If dispersal of *P. minutus* and *P. microps* along the coasts of the northeastern Atlantic and the North Sea is primarily controlled by passive larval displacement along oceanic currents, then their genetic structure should be concordant with a pattern of isolation-by-distance and both species should show a similar degree of genetic differentiation on the same scale. If the pattern of genetic differentiation shows no congruence with prevailing currents, then additional ecological, behavioural or historical factors may be needed to explain population structure.

The Baltic Sea

The Baltic Sea is a shallow, non-tidal enclosed environment with estuarine-brackish water. This is due to a large input of freshwater from river runoff and a limited inflow of seawater from the adjacent North Sea via the Kattegat, which is mainly driven by meteorological events (Hinrichsen & Wulff, 1998). Brackish surface water flows out of the Baltic Sea into the North Sea and saline North Sea water flows in at depth through the Danish Straits. Thus, a stratification with saline bottom water and brackish surface water is achieved (Lass *et al*, 2001). Residence time of the water is about 35- 40 years. The average salinity is a third of the salinity of the adjacent North Sea, decreasing towards the northeast. A permanent halocline results in oxygen-poor deeper water masses, which are ventilated only by the occasional influx of oxygen-rich water from the adjacent North Sea (MacKenzie *et al*, 2000). Furthermore, northern parts of the Baltic Sea (Gulfs of Bothnia, Finland and Riga) are covered with ice during several months of the year. This combination of a low salinity and ice cover results in harsh conditions for the marine species, which are living at the edge of their salinity and temperature tolerance. Janson (1978) points out several conditions to which Baltic populations correspond: (1) they are able to withstand low salinities, (2) Baltic specimens are smaller in size and less specialised than their marine conspecifics and (3) biodiversity in the Baltic is lower than in marine systems.

The present Baltic Sea is very young, geologically speaking. During the last glaciation (Weichselian) the Baltic basin was covered with ice and a large meltwater lake was formed upon deglaciation, which constituted the first stage of the Baltic Sea (Andrén *et al*, 2002). This huge lake (the Baltic Ice Lake) was formed about 12 ka BP. During the following Yoldia Sea stage a temporary connection with the North Sea was established around 10 ka BP, when a large inflow of seawater took place, allowing euryhaline marine species to invade the area from the west. Subsequently, the sea connection was closed and a second lake period began (Ancylus Lake). The present connection with the North Sea via the Kattegat dates from only 8 ka BP (Björck, 1995).

The Mediterranean Sea

The Mediterranean Sea and the Atlantic Ocean are connected via the narrow entrance of the Strait of Gibraltar, where a surface inflow of less saline Atlantic water into the Mediterranean is complemented by an outflow of saline Mediterranean water at depth across the sill of Gibraltar

(Krijgsman, 2002). The surface 'collision' between the colder Atlantic and the warmer Mediterranean seawater is situated in the Alboran Sea, the most western part of the Mediterranean Sea. This results in a frontal zone, the Almeria-Oran Front (Tintore *et al*, 1988) associated with a persistent gyre. Apparently, the Almeria-Oran Front acts as a barrier for dispersal in a number of marine species (Zane *et al*, 2000; Jaziri & Benazzou, 2002; Ríos *et al*, 2002) and may limit gene flow in *Pomatoschistus sp* as well. However, in other species sufficient gene flow occurs to maintain genetic homogeneity between Atlantic and Mediterranean populations (Magoulas *et al*, 1996; Pujolar *et al*, 2002).

The Mediterranean Sea consists of two main basins, the eastern and the western Mediterranean basins, which are connected by the Strait of Sicily. The eastern and western basin comprise several sub-basins and regional seas, separated by straits and channels. The Sicily Strait is considered a biogeographical boundary between the eastern and western basin, and restricted gene flow between populations from the eastern and western basin has been reported for several species (Borsa *et al*, 1997a; Bahri-Sfar *et al*, 2000). In general, within-species differentiation is higher in the eastern than in the western basin, which is attributed to the differences in hydrography (Bahri-Sfar *et al*, 2000). Incoming Atlantic water flows eastward along the coast of northern Africa (Algerian current) and branches off along the coast of Tunisia (Strait of Sicily) towards the north along the coasts of Sicily and Italy, resulting in a large cyclonic gyre across the western basin (Millot, 1999). This gyre might act as a means for larval transport within the western Mediterranean Sea and, on the other hand, as a barrier for dispersal of marine organisms across the Sicily Strait. Circulation within the eastern Mediterranean Sea is limited and the northern parts of sub-basins such as the Aegean and the Adriatic Sea are isolated from the rest of the Mediterranean. This is reflected in the genetic structure of resident fish populations (Magoulas *et al*, 1996; Borsa *et al*, 1997b).

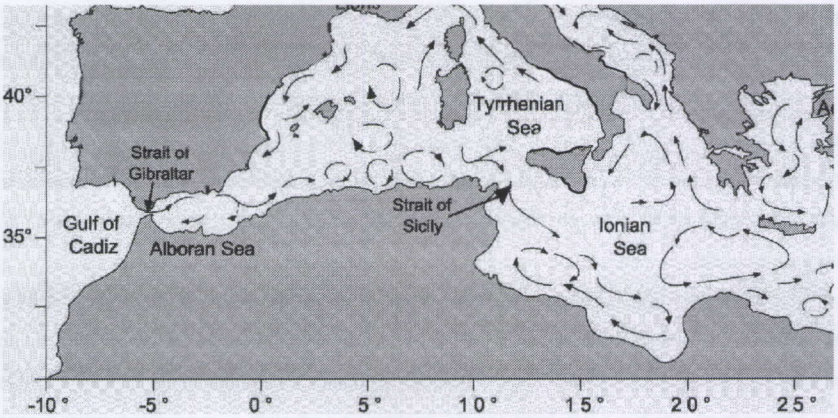

Fig. 1.4: Present circulation patterns in the Mediterranean Sea. After Cacho *et al* (2002).

Geological data on the Mediterranean Sea reveal evidence for a period of desiccation between 5.96 and 5.33 Ma BP due to closing of the connection with the Atlantic Ocean, the so-called Messinian salinity crisis (Hsü *et al*, 1977). The Mediterranean Sea was reduced to a series of hyper-and hyposaline lakes in the various basins and most of the ancestral fauna got extinct. After reflooding of the Mediterranean Sea through the newly formed Strait of Gibraltar, Atlantic marine fauna and flora invaded the Mediterranean Sea. It has been postulated that the Messinian salinity crisis induced the speciation of the freshwater gobies, which are endemic to the Mediterranean Sea (Miller, 1990), but phylogenetic analysis of mtDNA failed to confirm this (Penzo *et al*, 1998) and speciation patterns within the Atlanto-Mediterranean gobies are complex (Huyse, 2002). At present, both the time frame and the events triggering the speciation of these gobies remain unclear.

3.3.3. Small-scale genetic structure and diversity of Pomatoschistus sp.: selective pressure along an environmental gradient?

The influence of environmental heterogeneity on intraspecific genetic variation has been the subject of a long-lasting debate with conflicting results. For example, a significant correlation has been

established between the level of within-species genetic variability and the degree of environmental heterogeneity characterising the habitat. Species living in a heterogeneous environment tend to display higher levels of genetic diversity (Powell, 1971; Levinton & Suchanek, 1978). This has been explained as the effect of balancing selection, with differential fitness for the respective genotypes in the various niches provided by a heterogeneous environment ('niche-width variation hypothesis'). In contrast, Qian & Davies (1996) reported a low degree of genetic diversity among organisms in habitats displaying a high degree of heterogeneity. They suggested that a higher level of phenotypic plasticity might compensate for the lack of genetic variation and thus allow for adaptation to changing environments.

Wallis & Beardmore (1984b) reported within the genus *Pomatoschistus* clear differences in the degree of heterozygosity that were significantly correlated with heterogeneity in the habitat in which the respective species preferentially occur. Estuarine *Pomatoschistus sp.* displayed a significantly higher level of variation than marine gobies, but it was unclear whether this was due to either heterosis or else diversifying selection because of habitat patchiness and different fitness for the various genotypes. In contrast, Sorice & Caputo (1999) did not observe any difference in habitat preference between genetically highly or less variable Mediterranean goby species. The differences in habitat heterogeneity in the Schelde estuary and on the sand banks of the Belgian Continental Shelf make this area well-suited for assessing the influence of environmental heterogeneity on the small-scale genetic structure and diversity of *Pomatoschistus* sp. These species occur commonly in the estuarine, coastal and off-shore habitats on the Belgian Continental Shelf (Hamerlynck & Cattrijsse, 1994) and the Schelde estuary (Maes *et al*, 1998b).

Hence, the aim of this study is threefold:

(1) to interpret patterns of genetic structure in *P. minutus* and *P. microps* throughout their distributional range, using available data on historical and recurrent oceanographical features (Chapter II and III).

(2) to assess the influence of dispersal and environmental variables on a regional scale (i.e. the Belgian Continental Shelf and the Schelde estuary) for the sand goby, *Pomatoschistus minutus*, and Lozano's goby, *P. lozanoi*, (Chapter IV).

(3) to compare genetic patterns of a coastal-estuarine and a coastal-marine goby for assessing the influence of ecological and behavioural factors on population structure (Chapter V).

CHAPTER II

GENETIC STRUCTURE AND PHYLOGEOGRAPHY OF THE SAND GOBY, *POMATOSCHISTUS MINUTUS* (PALLAS, 1770), THROUGHOUT ITS DISTRIBUTIONAL RANGE

PAPER I

Allozyme variation and genetic divergence in the sand goby, *Pomatoschistus minutus* (Teleostei: Gobiidae)

ABSTRACT

Samples of the widely distributed sand goby *Pomatoschistus minutus* have been investigated genetically from seven localities in the North-eastern Atlantic Ocean, the North Sea, the western Mediterranean Sea and the Adriatic Sea. Levels of genetic diversity and differentiation were assessed with cellulose acetate (CAGE) gel electrophoresis for nine enzyme systems. Pair-wise F_{ST} values between samples point to a reduction or even absence of gene flow between the Adriatic and the other samples, including the western Mediterranean Sea (multi-locus $F_{ST} = 0.321$; multi-locus $G_{ST} = 0.287$). The differentiation between the Adriatic sample and all the others was of the same order of magnitude as between *P. minutus* and its closest relative, *P. lozanoi.* The sample from the Adriatic Sea was differentiated from the other samples mainly by allele frequency differences at the lactate dehydrogenase loci *LDH-A** and *LDH-C** (CAGE). At the sampling sites outside the Adriatic Sea the sand goby shows the typical features of a marine fish with a high level of gene flow and a low degree of genetic differentiation, which is roughly ten times lower than when the Adriatic *P. minutus* are included (multi-locus $F_{ST} = 0.019$; multi-locus $G_{ST} = 0.028$).

INTRODUCTION

The sand goby *Pomatoschistus minutus* (Pallas, 1770) is one of the most abundant and widespread *Pomatoschistus* species in the northeastern Atlantic Ocean. Its distributional range extends from the

north of Norway (Tromsø) and the Faroe Islands (Miller, 1986 and references herein), to the coasts of Western Europe and the British Isles, the Baltic Sea, the Mediterranean Sea and the Black Sea. Although small in size and not commercially exploited, their ecological importance can hardly be overestimated considering the fact that *Pomatoschistus* species play a major role in coastal ecosystems. Consequently, a large number of studies on various aspects of *P. minutus* population dynamics, behaviour and ecology have been carried out (e.g. Aarnio & Bonsdorff, 1993; Hamerlynck & Cattrijsse, 1994; Forsgren, 1997; Zander *et al*, 2002).

Miller (1986) reports a geographically distinct subspecies, *P. minutus elongatus* (Canestrini, 1861) for the Mediterranean and Black Sea, differing from *P. minutus minutus* in the northeastern Atlantic Ocean. *P. m. elongatus* is morphologically distinguished from *P. m. minutus* by a dark chin spot in the females and breast pigmentation in both sexes. Ecological differences between Atlantic and Mediterranean sand gobies have also been described (reviewed in Bouchereau & Guelorget, 1997). Atlantic and Mediterranean *P. minutus* perform a spawning migration towards the coast during the breeding season, although sedentary *P. minutus* living in Mediterranean lagoons have been documented. The reproductive season of *P. minutus* is in winter, lasting from December to April, while they reproduce during spring in the Atlantic Ocean. Contrary to Mediterranean sand gobies, Atlantic *P. minutus* carry also out a thermal migration in winter into deeper water, when water temperature drops below 3-5°C (Fonds, 1973).

Whether this morphological and ecological differentiation is the result of phenotypic plasticity in diverse habitats, or the result of a genuine reproductive isolation has not been documented. Wallis & Beardmore (1984a, b) carried out an extensive allozyme survey of several species belonging to the gobiid genera of *Pomatoschistus, Knipowitischia* and *Neogobius* at 31 loci, but with the only aim to assess the robustness of morphology-based systematic relationships between the species. No geographically distinct populations within species were screened. The only allozyme survey of geographically distinct sand gobies so far has been carried out on samples from Bristol Channel and the Lagoon of Venice (Wallis & Beardmore, 1983; Stefanni *et al*, 1996). In these studies, clear allozymatic differences between *P. minutus* from the lagoon of Venice and the Atlantic Ocean were recorded at several allozyme loci. Wallis & Beardmore reported nearly fixed allele frequency differences at loci *LDH-A**, and *LDH-C**. Moreover, they claimed to have found a fourth *LDH* locus in the Venetian sample, and called it *'LDH-0'*, which would be unique in this species. They suggested that this extra locus would be an adaptation to the specific environment of the highly variable and polluted lagoon of Venice. However, Stefanni *et al* (1996) found no

indications of this putative fourth locus and no indications of differentiation at locus *LDH-C** either. Few genetic data of other Mediterranean samples of *P. minutus* are available. Thus, it is unknown if the Venetian population is representative of the Mediterranean sand gobies or whether they represent a distinct population, which has been isolated in the Adriatic Sea. Reduced gene flow between Atlantic and Mediterranean populations of several fish species has been recorded (e.g. Allegrucci *et al*, 1997; Borsa *et al*, 1997b; Roldán *et al*, 1998; Lundy *et al*, 1999), but in others a sufficient amount of gene flow occurs to prevent differentiation (Magoulas *et al*, 1996; Pujolar *et al*, 2002).

In the present work we have compared nine enzyme systems of *P. minutus* from the northeastern Atlantic Ocean, the North Sea, the western Mediterranean Sea (Gulf of Lions) and the Adriatic Sea employing cellulose acetate gel electrophoresis (CAGE), in order to answer following questions: (1) to which extent are the observed differences between geographical populations the result of restricted gene flow rather than phenotypic plasticity? (2) Is there any genetic basis for assigning subspecies status to the Mediterranean *P. minutus (elongatus)*? (3) What is the taxonomic status of the Venetian *P. minutus*?

MATERIALS AND METHODS

Samples

A total of 335 specimens of *P. minutus* from seven localities was subjected to allozyme electrophoresis (Fig. 2.1.1, Table 2.1.1). Samples were collected by beam trawl or hand net, frozen for transport and stored at - 80°C. Individuals were identified morphologically by their suborbital papillae patterns (Miller, 1986). Morphological identification was confirmed using the isozyme pattern of creatine kinase (*CK-A**). *P. minutus* has a slower moving allele at locus *CK-A** compared to *P. lozanoi* (Wallis & Beardmore, 1984a).

Table 2.1.1. Sampling localities for *P. minutus* and number of individuals used for allozyme electrophoresis (N).

Sampling site	Latitude	Longitude	Code	N
Bergen (North Sea, Norway)	60° 23' N	5° 21' E	Ber	59
Oban (Atlantic Ocean, UK)	56° 24' N	5° 28' W	Oba	64
Frisian Front (Wadden Sea, The Netherlands)	53° 30' N	4 ° E	FrF	38
Texel (Wadden Sea, The Netherlands)	53° 00' N	4° 46' E	Tex	36
Ostend (North Sea, Belgium)	51° 13' N	2° 54' E	Ost	52
Venice (Adriatic Sea, Italy)	45° 26' N	12 °19' E	Ven	50
Pérols (Gulf of Lions, France)	43° 34' N	3° 57' E	Per	72

Figure 2.1.1. Sampling sites of *P. minutus*

Cellulose acetate gel electrophoresis

Liver, eye and muscle tissue were ground in 50-150 µl distilled water. The samples were centrifuged for 3 min and the supernatants subjected to electrophoresis (Richardson *et al,* 1986), using Titan III cellulose acetate gels (Helena Biosciences). Run time varied between 25 and 40 min depending on mobility of the alleles at the respective loci, and was carried out at 220 V and 400 mA at room temperature. Two buffer systems, tris-maleate at pH 7.8 and tris-glycine at pH 8.8, were used as described by Hebert & Beaton (1989). Staining was carried out according to Hebert & Beaton (1989). A sample of *P. lozanoi* from the Belgian coast consisting of 42 fish was subjected to cellulose acetate electrophoresis as well and used for comparison; the same loci were screened as for *P. minutus*.

A total of nine enzyme systems, corresponding to 15 loci, was used as a basis for genetic comparisons. The slowest migrating locus was termed 1 or A and nomenclature follows Shaklee *et al* (1990). For each locus allele mobility was calculated with reference to the most common allele, which was assigned a mobility of 100. The values for allele frequencies, percentage polymorphism and observed and expected heterozygosity are listed in Appendix 2.1.

Table 2.1.2. Enzymes and their Enzyme Commission (E.C.) numbers and tissue employed. M = skeletal muscle; E = eye; L = liver.

Enzyme	Locus (tissue)
Adenylate kinase (AK) [EC 2.7.4.3]	AK* *(M)*
Aspartate aminotransferase (AAT) [EC 2.6.1.1]	*AAT** (M)
Creatine kinase (CK) [EC 2.7.3.2]	*CK-A** (M)
Fumarate hydratase (FH) [EC 4.2.1.2]	*FH** (M)
Glucose-6-phosphate isomerase (GPI) [EC 5.3.1.9]	*GPI-A** (M,E), *GPI-B** (M, E)
Isocitrate dehydrogenase (IDHP) [EC 1.1.1.42]	*IDHP-1** (M), *IDHP-2** (L)
L-Lactate dehydrogenase (LDH) [EC 1.1.1.27]	*LDH-A** (M), *LDH-B** (M), *LDH-C** (E)
Malate dehydrogenase (MDH) [EC 1.1.1.37]	*MDH-A** (M), *MDH-B** (M)
Phosphoglucomutase (PGM) [EC 5.4.2.2]	*PGM-1** (M), *PGM-2** (M)

Data analysis

Genetic diversity was measured as the level of polymorphism, observed and expected heterozygosity and haplotype diversity. Allele frequencies and the observed and unbiased expected heterozygosity were calculated in GENETIX version 4.02 (Belkhir *et al,* 2001). Linkage disequilibrium and Hardy-Weinberg equilibrium were tested for significance in GENEPOP version 3.1 (Raymond & Rousset, 1995). F-statistics were calculated according to Weir & Cockerham (1984) in GENETIX. Standard deviations of single-locus F_{ST} values were obtained by jacknifing over all samples according to Weir (1990) and the significance of the multi-locus F_{ST} values was assessed with permutation tests (1000 replicates). Multi-locus G_{ST} (Nei, 1973) across all samples was also calculated in GENETIX. A Multidimensional Scaling Analysis (MDS) was carried out on pair-wise F_{ST} values in the program STATISTICA version 6.0 (STATSOFT, 2001) for assessing whether any grouping structure could be detected. For comparison pair-wise Nei´s genetic distances (Nei, 1978) and Cavalli-Sforza & Edwards´ (1967) chord distances were computed and significance was assessed with permutation tests in GENETIX (1000 replicates). Isolation by distance was assessed with a Mantel test (Mantel, 1967) in GENETIX and by plotting $F_{ST}/(1-F_{ST})$ against the logarithm of the geographic distance according to Rousset (1997). Geographical distances were measured as the shortest coastal distances between sites in software package ENCARTA (Microsoft, 2001).

RESULTS

Allele frequencies, Hardy-Weinberg equilibrium and linkage disequilibrium

Ten loci out of 15 were polymorphic. Frequencies of the most common allele were generally around 0.99, with the exception of *LDH-C*, PGM-1** and *PGM-2*,* which were polymorphic at the 0.95 level in most samples. Values for allele frequencies, proportion of polymorphic loci and heterozygosity are presented in Appendix 2.1. The lowest level of observed heterozygosity was recorded in the Oban sample (H_o = 0.040). The samples from Bergen, Pérols and Ostend showed the highest values for heterozygosity (H_o = 0.084, 0.082 and 0.080 respectively). The highest mean number of alleles was recorded in the sample from Pérols (1.80) while the lowest value was

recorded in the Oban sample (on average 1.53 alleles per locus). The lowest proportion of polymorphic loci was observed in Oban ($P_{0.99} = 0.20$), Ostend and Bergen ($P_{0.99} = 0.27$), whereas the highest value occurred at Texel ($P_{0.99} = 0.48$). Fisher's test for linkage disequilibrium across all samples was not significant. In individual samples only two significant cases ($P < 0.05$) of linkage disequilibrium were observed. At Pérols, *LDH-C** was linked with *PGM-2**. In the sample from Texel linkage disequilibrium between *LDH-C** and *PGM-1** was recorded.

F-statistics calculated per locus across all samples revealed a significantly positive F_{IS} value at locus *LDH-C** (Table 2.1.3), indicating departures from Hardy-Weinberg equilibrium due to a heterozygote deficit. Single-locus F_{IS} values at each of the localities (not listed) revealed a heterozygote deficit in all samples with the exception of the Venetian sample. The F_{IS} value for *IDHP-2** is also significant, but this is due to one homozygote individual (*IDHP-2*86/86*) in the sample from the Frisian Front, where only 12 fish stained positive for this locus. F_{ST} values across all samples were significantly positive for loci *LDH-C*, LDH-A** and *PGM-2** (Table 2.1.3).

Table 2.1.3: Single-locus and multi-locus F-statistics (Weir & Cockerham, 1984) over all samples per locus for the polymorphic loci. Values significant by permutation tests are indicated with an *.

Locus	F_{IS}	F_{ST}	F_{IT}
*AAT**	-0.039	0.040	0.001
*GPI-B**	-0.028	0.017	-0.010
*IDHP-2**	0.656*	0.036	0.668*
*LDH-A**	0.065	0.821*	0.832*
*LDH-B**	-0.003	-0.003	-0.007
*LDH-C**	0.327*	0.265*	0.505*
*MDH-A**	-0.003	0.001	-0.002
*MDH-B**	-0.005	0.004	-0.001
*PGM-1**	0.039	-0.003	0.037
*PGM-2**	0.146	0.146*	0.271*
Multi-locus	0.185*	0.321*	0.405*

Allozyme patterns between samples

Allozyme patterns for most of the loci investigated are similar for all geographic samples with the exception of *LDH-A*, LDH-C** and *PGM-2** in the Venetian sample compared to the others. Exact tests for differentiation in allele frequencies at individual loci between samples confirmed that most significant differentiation was due to differences at loci *LDH-A*, LDH-C** and *PGM-2** (Table 2.1.4). Whereas almost all samples of *P. minutus* are close to fixation for *LDH-A*100*, with a frequency ranging from 0.972 (Frisian Front) to 1.000 (Bergen), this allele was rare in the Venetian P. minutus, occurring in a frequency of only 0.092 (Appendix 2.1). At locus *LDH-C**, allele *LDH-C*100* is absent from the Venetian sample, whereas it is the most common allele in all other samples, with frequencies ranging from 0.482 (Pérols) to 0.773 (Bergen). On the other hand, the Venetian sample is almost fixed for allele *LDH-C*115*, with a frequency of 0.978. Thus, apparently allele frequencies are inversed at the loci *LDHA** and *LDH-C** for the Venetian samples compared to all the others. Referring to Wallis & Beardmore (1983), we found no indications of a fourth locus, the so-called *'LDH-0*'* in the Venetian sample. We did notice however, an extra zone of activity in degraded samples near the cathode, which may suggest that this putative locus could be an artefact due to tissue degradation. At locus *PGM-2** the Venetian sample shows a higher frequency in the occurrence of allele *PGM-2*100* compared to the others (excluding the Oban sample due to its low sample size); in Venice, allele *PGM-2*100* occurs at a frequency of 0.852, while frequencies in the other samples range from 0.292 to 0.561. Allele *PGM-2*106*, which is common in all other samples (except Oban) with frequencies from 0.268 to 0.625, is only rarely found in the Venetian sand gobies (frequency of 0.034). The sample from Oban did not show any polymorphism at this locus, resulting in a significant differentiation with the other samples (Table 2.1.4). However, only 10 fish of this sample stained positively at *PGM-2**. Thus, this significant effect may as well be caused by sampling bias.

Table 2.1.4. *Pomatoschistus minutus*: P-values for the significant differences among the pair-wise comparison of allele frequencies at the various loci based on CAGE.

Locus	Sample 1	Sample 2	P-value	Locus	Sample 1	Sample 2	P-value
*LDH-A**	Ber	Ven	0.000	*PGM-2**	FrF	Ven	0.000
	FrF	Ven	0.000		Ber	Ven	0.000
	Tex	Ven	0.000		Tex	Ven	0.000
	Oba	Ven	0.000		Per	Ven	0.000
	Per	Ven	0.000		Ost	Ven	0.000
	Ost	Ven	0.000		Ber	Per	0.000
					FrF	Per	0.014
*LDH-C**	FrF	Ven	0.000		Oba	Per	0.004
	Tex	Ven	0.000		Oba	Ost	0.000
	Oba	Ven	0.000		Oba	FrF	0.000
	Per	Ven	0.000		Oba	Ber	0.000
	Ost	Ven	0.000	*GPI-A**	Ber	Oba	0.005
	Tex	Per	0.003		Ber	Per	0.007
	Oba	Per	0.000	*AAT**	Oba	Ven	0.028
	Ber	Per	0.000		Ost	Ven	0.038
	Oba	Ost	0.040	*IDHP-2**	Ber	FrF	0.037

Most but not all rare alleles are shared among all samples (Appendix 2.1). Even in the distinct Venetian sample no exclusive alleles were found. For example, allele *AAT*150* is confined to the samples from Venice and Pérols. Allele *GPI-B*116* is found in the samples from Venice, Bergen and Texel. Allele *LDH-A*-60* is shared by the Ostend, Frisian Front and Venice samples. On the contrary, allele *GPI-B*125* and *PGM-1*73* are unique to the Bergen sample, while *IDHP-2*115* and *LDH-C*-120* were only recorded in Oban.

Genetic structure

The highest degree of genetic differentiation was recorded between the Venetian sample and all the others, including the western Mediterranean Sea (Table 2.1.5). A comparison with the closest relative of *Pomatoschistus minutus*, Lozano's goby (*Pomatoschistus lozanoi* de Buen, 1923), shows that the differentiation between the Adriatic and the other sand gobies is of the same order of

magnitude as between *P. minutus* and *P. lozanoi* (Table 2.1.5): pair-wise multi-locus F_{ST} between the Venetian and other sand gobies calculated over all loci range from 0.582 to 0.697, while the pair-wise F_{ST} values between the other sand goby samples are at least tenfold lower, ranging from – 0.004 (Texel - Bergen) to a maximum of 0.057 (Oban - Pérols). The F_{ST} value between *P. lozanoi* and the various samples of *P. minutus* amounts to 0.78 (Table 2.1.5). We notice that the sample from Pérols is most closely related to the Venetians, as reflected in the slightly lower values for pair-wise F_{ST} (Table 2.1.5) and genetic distances (Table 2.1.6).

Multi-locus F_{ST} is about tenfold lower when the Adriatic *P. minutus* are excluded from the analysis (F_{ST} = 0.321 and F_{ST} = 0.019 respectively). Multi-locus G_{ST} values show a similar pattern, amounting to 0.287 for all samples and to 0.028 without the Adriatic sample. Little differentiation is recorded between samples from the western Mediterranean Sea (Pérols) and the southern North Sea (Ostend). However, the samples from Bergen, Oban and Texel are differentiated from the western Mediterranean Sea. This is also shown on the MDS, where the sample from Pérols is presented separately from all the others (Fig. 2.1.2). Within the Atlantic basin, genetic differentiation is also low and always non-significant. Nevertheless, the MDS shows some differentiation within the Atlantic basin (Fig. 2.1.2). The sample from Oban clusters together with the Bergen sample; these are separated from the southern North Sea samples. The relatedness between these two samples is due to similar frequencies of allele *LDH-C*100* (Appendix 2.1), which are higher than in the other samples (0.733 and 0.721 in Bergen and Oban respectively, compared to a range from 0.482 to 0.578 for the others).

Table 2.1.5. Pair-wise estimates of multi-locus F_{ST} (Weir and Cockerham, 1984), calculated over all loci except for *PGM-2**. Significance: (*) P<0.05, (**) P<0.01 and (***) P<0.001 (1000 permutations). For abbreviations see Table 2.1.1. *P. loz* refers to pair-wise multi-locus F_{ST} values between the *P. minutus* samples and a sample of *P. lozanoi* .

	Ber	**FrF**	**Tex**	**Oba**	**Per**	**Ven**	**P. loz**
Ost	0.023	-0.036	-0.002	0.007	0.005	0.612***	0.670***
Ber		0.035	-0.004	-0.003	0.077***	0.702***	0.799**
FrF			0.004	0.017	0.000	0.601***	0.768***
Tex				-0.005	0.037**	0.657***	0.784***
Oba					0.057***	0.676***	0.792***
Per						0.585***	0.780***
Ven							0.873***

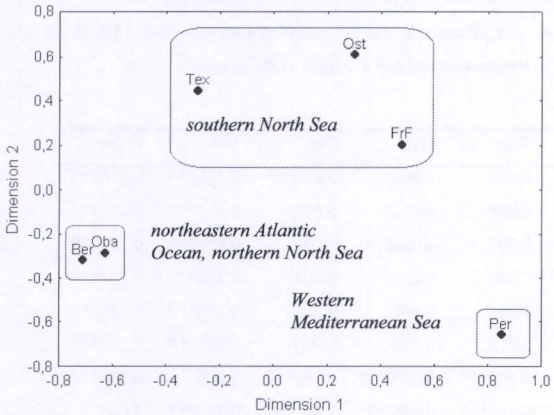

Fig. 2.1.2: Multidimensional Scaling Analysis on pair-wise F_{ST} values. Stress value = 0.000. The Venetian sample is excluded to avoid distortion, due to its large differentiation from all other samples.

Values of pair-wise genetic and chord distances (Nei, 1978 and Cavalli-Sforza & Edwards, 1967, respectively) yielded comparable results with respect to degree of population differentiation, (1) with the highest amount of differentiation between the Venetian sand gobies and all the others and (2) comparable to the genetic distance between *P. minutus* and *P. lozanoi* (Table 2.1.6). The only other significant differences were recorded between the samples from Pérols and Bergen. A Mantel test did not show any correlation between geographic and Nei's genetic distance (Pearson coefficient = 0.61, n.s.). Nevertheless, the plot of geographic distance versus $F_{ST}/(1-F_{ST})$ shows some indications for an isolation-by-distance effect (Fig.2.1.3). In the MDS we notice also that the samples are separated on a geographical gradient, with the northernmost (Bergen) and southernmost (Pérols) samples on the extremes of Dimension 1, while the North Sea samples are situated in between (Figure 2.1.2).

Table 2.1.6. *Pomatoschistus minutus*: pair-wise genetic distances (Nei, 1978) (above diagonal) and Cavalli-Sforza & Edwards (1967) (below diagonal) chord distances for all loci except *PGM-2**. Significance: (*) P<0.05, (**) P<0.01 and (***) P<0.001 are based on 1000 permutations. For abbreviations of the sampling sites see Table 2.1.1. *P. loz* refers to pair-wise multi-locus F_{ST} values between the *P. minutus* samples and a sample of *P. lozanoi* .

	Ber	**Oba**	**FrF**	**Tex**	**Ost**	**Per**	**Ven**	***P. loz***
Ber	0.000	0.000	0.003	0.000	0.002	0.006***	0.121***	0.216***
Oba	0.018	0.000	0.001	0.000	0.001	0.004	0.113***	0.222***
FrF	0.025	0.022	0.000	0.001	-0.001	0.000	0.095***	0.235***
Tex	0.009	0.010	0.014	0.000	0.000	0.003	0.110***	0.224***
Ost	0.013	0.013	0.013	0.006	0.000	0.001	0.099***	0.230***
Per	0.025***	0.016	0.016	0.012	0.010	0.000	0.085***	0.236***
Ven	0.243***	0.221***	0.190***	0.209***	0.205***	0.163***	0.000	0.394***
P.loz	0.467***	0.477***	0.501***	0.480***	0.489***	0.487***	0.743***	0.000

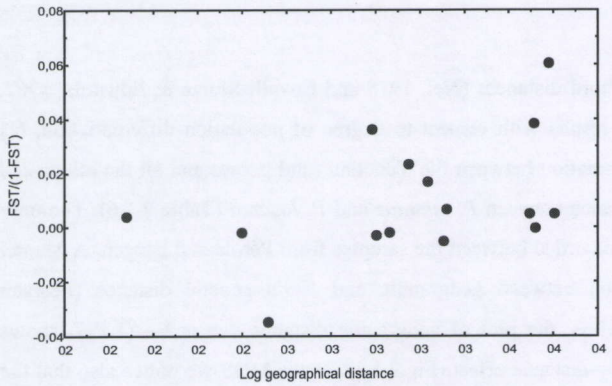

Fig. 2.1.3: Scatterplot of genetic differentiation measured as $F_{ST}/(1-F_{ST})$ versus log geographic distance. The Venetian sample is excluded.

DISCUSSION

Genetic diversity, Hardy-Weinberg equilibrium and linkage disequilibrium

Genetic variability, measured as the average number of alleles, expected heterozygosity and degree of polymorphism, varied among samples. In the case of *LDH-C** in particular, little or no polymorphism at this locus has been reported using SGE (Wallis & Beardmore, 1984a, Stefanni *et al*, 1996), with the exception of the Venetian sand gobies, where Wallis & Bearmore (1983) reported fixation for a faster moving allele than in the Atlantic *P. minutus*. Our CAGE results show a distinct polymorphism in all samples with three commonly occurring alleles *(LDH-C*100, LDH-C*107, LDH-C*115*) and two rare alleles (*LDH-C*90 and LCH-C*120*). A possible explanation for this discrepancy could be the weak response to staining of this locus on SGE.

Most samples are in Hardy-Weinberg equilibrium after sequential Bonferroni correction for all polymorphic loci, with the exception of locus *LDH-C**. Allele frequencies at locus *LDH-C** reveal a significant heterozygote deficit, which may be due to various reasons. One possible explanation could be the Wahlund effect, i.e. the mixing of different subpopulations, but then we should notice the same effect at the other polymorphic loci, which is not the case. Another possibility could be related to the occurrence of null alleles, which would cause a heterozygote to be identified as a homozygote (Richardson *et al,* 1986), but this remains unproven. The presence of null alleles may be detected by comparing the intensity of the hybrid bands between the distinct loci among individuals. A hybrid band with a lower intensity compared to other individuals may point to a null allele at the locus under study. However, in the case of *P. minutus* hybrid bands between the *LDH* loci were always quite weak. Hence, comparing hybrid bands between individuals did not yield any consistent results. Another explanation might be found in selective mortality of heterozygotes (Zouros & Foltz, 1984) but detailed biochemical studies would be required to support this hypothesis (DiMichele *et al,* 1991). Lactate dehydrogenase is one of the enzymes that is known to be under selective pressure (Powers & Schulte, 1998). However, regardless of the mechanism behind the *LDH-C** heterozygote deficit, the distinction between Venetian and Atlantic samples remains with or without including locus *LDH-C**.

Genetic structure and gene flow in *Pomatoschistus minutus*

Most striking in our results is the distinctness of the Venetian *P. minutus* as opposed to the lack of differentiation within the Atlantic sand goby samples. In the review of Ward *et al* (1994) an average multi-locus G_{ST} value (Nei, 1973) of 0.062 was found for marine fish. This is about tenfold lower than the value we obtain across all *P. minutus* samples. Excluding the Adriatic sample we found a multi-locus G_{ST} of only 0.028, which is comparable to the average for marine fish as reported by Ward *et al* (1994).

The status of the Venetian P. minutus: allopatric speciation or natural selection?

The highest level of differentiation between Venetian and other sand gobies is found at the loci *LDH-A** and *LDH-C** and, although to a lesser extent, at *PGM-2**, as also reported by Wallis & Beardmore (1983). Wallis & Beardmore (1983) noticed that the distinct allele for *Pomatoschistus minutus* in the Venice lagoon at locus *LDH-A** showed the same electrophoretic mobility as in the related estuarine species *Pomatoschistus microps* (Krøyer, 1838) and suggested this distinct allele being an adaptive response to environmental pressures in a variable, enclosed environment. However, pair-wise genetic distances between the Venetian sample and all the others are of the same order of magnitude as between *P. minutus* and its closest relative, *P. lozanoi*, suggesting limited or probably absent gene flow with the western Mediterranean (Table 2.1.4). The observed differentiation has been found at three loci: *LDH-C*, LDH-A** and *PGM-2**. Moreover, results of sequence analysis of mtDNA indicate a significant differentiation in the D-loop between Venetian and other *P. minutus*, while little differentiation is observed between the other samples (Stefanni & Thorley, 2003; Gysels, unpublished results). While we do not argue on the fact that natural selection may be operating on locus *LDH-A**, we are inclined to support the existence of reproductive isolation between the Venetian sand gobies and the others. Hence, the large degree of differentiation between the Venetian *P. minutus* and the others arises questions about the taxonomic status of the Venetian (Adriatic) population. One could argue that the Venetian and other sand gobies share almost all their alleles (with the exception of *LDH-C*-100* which is absent in the Venetian sand gobies), suggesting that this degree of differentiation might be at the population level rather than at the species level. No exclusive alleles were found in this sample either. However,

examples of closely related species sharing all or almost all alleles are common in literature. For example, an allozyme survey of the African catfish species *Clarias gariepinus* and *C. anguillaris* revealed no diagnostic allozyme loci between these species among 25 loci screened; only highly significant differences in allele frequencies were recorded (Agnèse *et al*, 1997). Similarly, Cleary *et al* (2002) did not find any diagnostic locus among 16 loci screened between two *Colias* species. Feresu-Shonhiwa & Howard (1998) failed to find diagnostic loci between several species of *Oreochromis* sp. Thus, apparently, genetic differences between closely related species are not always reflected in the presence of diagnostic loci. Moreover, in our study we only screened 15 loci. The genome contains many more loci and it is not unlikely that other loci may be diagnostic for the Venetian *P. minutus*.

Whether the Venetian sand goby is reproductively isolated from the sand gobies inhabiting other parts of the Adriatic Sea would require further sampling along the Adriatic coast. *P. minutus* is not permanently present in the Venetian lagoon as it migrates towards the sea in winter (Gandolfi *et al,* 1991) and probably carries out a spawning migration towards sea in the spring as well, as described for all other *P. minutus* populations throughout the distributional range of the species. The northern Adriatic Sea is partially isolated from the rest of the Mediterranean Sea due to the presence of a large cyclonic gyre in the Southern Adriatic Sea (Artegiani *et al*, 1993). This has been proposed as a mechanism responsible for the differentiation observed in the Adriatic populations of other fish species (Magoulas *et al*, 1996). The fact that the Adriatic sand gobies seem more related to the other western Mediterranean Sea sand gobies than to the Atlantic *P. minutus* may point to a common ancestry, but for resolving these phylogenetic relationships a more informative marker such as mtDNA is required. Stefanni & Thorley (2003) suggest that a population of Mediterranean sand gobies migrated northward and got trapped in the cul-de-sac of the Adriatic Sea upon rising temperatures after the last glaciation event. They postulate that the sand gobies might be prevented from migrating south due to higher temperatures. Indeed, *P. minutus* not only avoids cold water, but thermal migrations into deeper water when temperature exceeds 19°C have been reported as well (Hesthagen, 1979). However, considering the large degree of differentiation with the other *P. minutus*, this split must predate the last deglaciation 10 ka BP.

Gene flow in the sand goby throughout its distributional range

Adult *Pomatoschistus minutus* are adapted to a demersal life-style, with the pelvic fins fused into a suction disc, and are considered poor swimmers (Miller, 1986). Hence, they have probably a small range of movement, migrations being limited to spawning and (in northern areas) thermal migrations. Eggs are demersal, and thus any large-scale gene depends mainly on the planktonic larval stage, which lasts about one month (Fonds, 1973). The extended pelagic larval phase provides them with a high potential for dispersal. The small pair-wise F_{ST} values between the samples from the western Mediterranean Sea, the English Channel, and the Wadden Sea point indeed to a high level of gene flow throughout the distributional range of the species. Yet, allozyme results showed indications for a possible population structuring within the Atlantic basin: (1) a slight effect of isolation-by-distance, which is not unexpected for a species relying on larval dispersal for covering large distances and (2) the separation of the southern North Sea samples (Texel, Ostend, Frisian Front) as opposed to those from the Atlantic Ocean and the northern North Sea (Bergen, Oban). As the resolution of allozymes for disclosing the shallow population structure in marine fish is limited, a more sensitive marker such as mtDNA or microsatellites may yield a better picture of the structure of Atlantic sand gobies. Numerous studies exist where a significant genetic differentiation among marine fish population was observed with DNA markers where allozymes revealed no differences (e.g. Nesbø *et al*, 1999; Shaw *et al,* 1999a, b).
Finally, our data do not support the differentiation of the sand goby in the Atlantic subspecies *P. minutus minutus* and the Mediterranean subspecies *P. m. elongatus* as proposed by Miller (1986).

CONCLUSIONS

The sand goby has in general a low degree of population differentiation throughout the sites sampled, especially in the northeastern Atlantic Ocean. This is not unexpected considering its extended pelagic larval stage and thus high potential for dispersal. However, some indications for a group structure within the Atlantic basin were found with the southern North Sea samples clustering together and distinct from the Atlantic coast of Scotland and the northern North Sea. A slight (although not significant) effect of isolation-by-distance was also recorded. A significant

differentiation exists between Atlantic and western Mediterranean samples, suggesting either a barrier for dispersal across the Strait of Gibraltar or else the effect of isolation-by-distance. However, the degree of differentiation is clearly not sufficient to warrant subspecies status to the Mediterranean *P. minutus*. The Venetian sand gobies are very distinct from all the others and their taxonomic status should be reconsidered, but a more sensitive marker is warranted for defining the taxonomic status of this population.

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PAPER II

Recurrent and historic gene flow of the sand goby *Pomatoschistus minutus* on the European Continental Shelf and in the Mediterranean Sea

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Running title: phylogeography of the sand goby

ABSTRACT

Phylogeographic patterns of the sand goby, *Pomatoschistus minutus* (Gobiidae, Teleostei) were studied by means of sequence and Single Stranded Conformational Polymorphism analysis of a 283 bp fragment of the cytochrome b locus in the mtDNA. A total of 228 individuals sampled at thirteen sites throughout the species' distributional range revealed a moderate level of diversity and a low, but significant level of overall genetic differentiation. The highest amount of population subdivision was detected between the samples from the northeastern Atlantic Ocean and the Mediterranean Sea, which was mainly due to differentiation between the *P. minutus* from the Adriatic Sea and all the other sand gobies, including the samples from the western Mediterranean Sea. Limited genetic differentiation with a weak pattern of isolation-by-distance was recorded in the western Mediterranean Sea, the Atlantic Ocean and the Baltic Sea. Phylogeographic analysis suggested a contiguous range expansion in the Atlantic and Baltic basins and evidence for a glacial refugium in the southern North Sea. Baltic samples showed evidence for lineage sorting by the presence of the most common Atlantic haplotype, with source genotypes from the North Sea. In the sand gobies

from the western Mediterranean Sea a high number of endemic haplotypes as well as the most common Atlantic haplotype were recorded in appreciable frequencies. We suggest that this might be explained by a secondary contact between different mitochondrial lineages, which evolved in allopatry.

Keywords: *Pomatoschistus minutus*, cytochrome b, phylogeography, genetic structure, gene flow

INTRODUCTION

The genetic structure of marine organisms has been shaped and remodeled by major forces such as (1) oceanic currents as a means for either dispersal or retention (Lessios *et al*, 1999; Stepien, 1999) and (2) past climatological and vicariance events (Bernardi *et al*, 1993; Bernatchez & Wilson, 1998). Disentangling the relative importance of each of these factors for explaining population structure in the marine environment remains a challenge, because it requires the integration of data from different fields such as paleoclimatology, geology, (pale)oceanography and the biology of the species. Most marine species have an extended pelagic larval phase, which enables them to cover large distances via oceanic currents. Consequently, marine organisms reveal in general a low degree of population differentiation (for a review, see Ward *et al*, 1994) and phylogeographic patterns are often less resolved, contrary to freshwater and terrestrial species. This is reflected by the paucity of studies on the phylogeographic patterns of the ichtyfauna, and in particular from the northeastern Atlantic Ocean (but see Wilke & Pfenniger, 2002). Although numerous studies have been dealing with evolutionary patterns in the anadromous salmonids (Osinov & Bernatchez, 1996; García-Marín *et al*, 1999; Verspoor *et al*, 1999; Consuegra *et al*, 2002), data on the phylogeography of genuine marine fish along the European Atlantic coasts are scarce.

The sand goby, *Pomatoschistus minutus* (Pallas, 1770) is one of the most abundant fish species along the Atlantic coasts of western Europe (Healey, 1971). It occurs in the Black Sea, the Mediterranean Sea and the northeastern Atlantic Ocean from the south of Spain to northern Norway (Tromsø) (Miller, 1986). It is present in estuaries as well as in the open sea (Claridge *et al*, 1985; Maes *et al*, 1998a) but spawns usually near the coast (Fonds, 1973). Male *P. minutus* display courtship behaviour and establish a territory, build a nest under an empty bivalve shell and court a

female (Fonds, 1973). A female deposits eggs, which are fertilised by the male, who protects and fans them during several weeks until they hatch. Upon hatching the larvae dwell in the plankton for at least one month before metamorphosing (Fonds, 1973). In northern regions *P. minutus* carries out a thermal migration towards deeper water when water temperature drops below 4-5 °C (Fonds, 1973). Adults are considered poor swimmers and are adapted to a demersal life, pelvic fins being fused into a suction disc (Miller, 1986).

Any passive large-scale larval dispersal of marine organisms, including P. minutus in the northeastern Atlantic Ocean is influenced by the oceanic currents. The most important of these are the North Atlantic Current (NAC) and the Shelf Edge Current (SEC), flowing northward along the western coasts of the British Isles towards the Norwegian trench. An arm branches off at the Shetland Islands and flows southward along the eastern English coast and eastward towards the Skagerrak (Turrell, 1992). Atlantic water enters the North Sea also via the English Channel, resulting in a northward flow through the English Channel (Fig. 2.2.1). Thus, if present-day gene flow between Atlantic *P. minutus* is maintained by passive larval drift via currents, we expect little heterogeneity throughout the northeastern Atlantic basin. The Mediterranean Sea is connected with the Atlantic Ocean through the Strait of Gibraltar, but the real encounter between Atlantic and Mediterranean water takes place in the Alboran Sea, at the Almería-Oran Front (Tintore *et al*, 1988). This front acts as a barrier for dispersal to several marine species (Zane *et al*, 2000; Ríos *et al*, 2002) and may limit gene flow in *P. minutus*. Preliminary genetic studies based on allozymes (Paper I) indeed point to a high amount of gene flow throughout the distributional range of the species, with some differentiation between the Atlantic Ocean and the western Mediterranean Sea. However, the resolution of allozymes for detecting population structure is limited, especially in highly vagile marine species. Thus, analysis of these samples with a more sensitive (DNA) marker, in combination with a larger number of sampling sites, will shed more light on the population genetic structure of this widely distributed goby.

A second major factor for explaining contemporary population structure are past geological and climatological events. The present distribution of *P. minutus* in the northeastern Atlantic Ocean is the result of a northward population range expansion after the last glaciation (Weichselian), which ended about 10 ka BP. During the Last Glacial Maximum (22 ka BP) an ice sheet covered Scandinavia and most of the British Isles (Lowe & Walker, 1997); the Southern Bight of the North Sea was dry (van der Molen & de Swart, 2001a), but a glacial lake has been proposed in the southern North Sea (cited in Verspoor *et al*, 1999). The Iberian peninsula and the Mediterranean

Sea have served as a southern refugium for many northern species (Bianchi & Morri, 2000; Consuegra *et al*, 2002), but additional refugia at the margins of the ice sheets in the southern North Sea and the Baltic region have been proposed (García Marin *et al*, 1999; Koljonen *et al*, 1999; Verspoor *et al*, 1999). After deglaciation the sea level rose and the ocean invaded the Southern Bight of the North Sea through the Strait of Dover (van der Molen & de Swart, 2001a). By 7.5 ka BP the present connection between the southern North Sea and the Atlantic Ocean was formed. The re-establishment of the North Atlantic Current 10 ka BP (Harland & Howe, 1995) allowed marine organisms to migrate northward from their southern refugia. Whether *P. minutus'* range expansion occurred from a southern refugium only, or if isolated populations managed to survive in the north along the ice sheets and recolonised formerly glaciated areas, may be inferred from the geographic distribution of the genetic variation (Templeton *et al*, 1995).

The only phylogeographic analysis of the sand goby so far has been carried out by Stefanni & Thorley (2003), but they focused mainly on the distinctness of the Adriatic *P. minutus*. They postulate the existence of a distinct Evolutionary Significant Unit (ESU) in the northern Adriatic Sea, while the Atlantic and western Mediterranean sand gobies would belong to a second ESU. They proposed a postglacial isolation in the northern Adriatic as recent as 5-10 ka. However, this conflicts with our allozymes results where we found a much larger degree of differentiation, suggesting even a reconsideration of its taxonomic status (Paper I). Moreover, no phylogeographic inferences about the Atlantic and Baltic *P. minutus* were made. Hence, we aim to (1) complement the existing phylogeographic and population genetic information on the sand goby, including more Atlantic and Baltic samples, and (2) assess the genetic differentiation between Venetian and other sand gobies by means of a second (mitochondrial) genetic marker in order to reinforce our conclusions regarding the hypothesis of allopatric speciation in the Adriatic Sea.

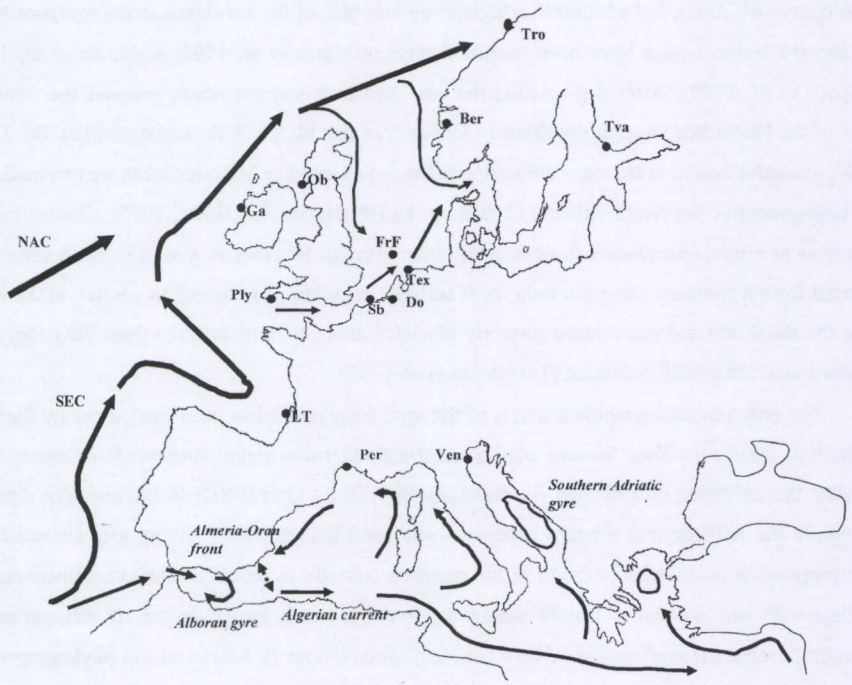

Figure 2.2.1. Sampling sites of P. minutus with main current patterns, compiled after Turrell (1992), Millot (1999) and Hansen & Østerhus (2000). Sampling sites are indicated with ● . Abbreviations of sampling sites are listed in Table 2.1.1. NAC: North Atlantic Current, SEC: Shelf Edge Current.

MATERIALS AND METHODS

Sequence and SSCP analysis of mtDNA

Samples were taken either by hand net, beach seine or beam trawling. Sampling sites with coordinates and number of fish screened are listed in Table 2.2.1. A total of 228 *Pomatoschistus minutus* was subjected to sequence and SSCP analysis. *Pomatoschistus* sp. were identified

morphologically on the basis of the dermal papillae of the head according to Miller (1986), and biochemically according to Wallis and Beardmore (1984a, b). Of each specimen a fin clip was taken for DNA extraction and stored in 100% non-denaturated ethanol. DNA extraction was carried out with the DNeasy tissue kit (Qiagen International) and with a standard phenol- choloroform protocol.

The universal cytochrome b primers (Kocher *et al*, 1989) did not work for *P. minutus*, but yielded a PCR product in the related species *Pomatoschistus lozanoi* (de Buen, 1923) and *Pomatoschistus microps* (Krøyer, 1838). Cytochrome b PCR products obtained for these two species were cloned with the TA cloning kit (Invitrogen) and both strands were sequenced with fluorescence labeled M13 primers on a LICOR 4200 automated sequencer (Westburg). A search in BLAST confirmed that the correct fragment had been amplified. Sequences of *P. microps* and *P. lozanoi* were aligned with cytochrome b sequences of other gobiid fishes retrieved from GENBANK (resp. *Tridentiger bifasciatus* AB021254, *Proterhorhinus marmoratus* AF082969 and *Rhinogobius giurinus* AB018997). A new set of primers was designed for amplifying a fragment of the cytochrome b locus, based on conserved sequences across these goby species. These primers amplified a 283 bp fragment in *P. minutus*, which was used in Single Stranded Conformational Polymorphism Analysis (SSCP) (Orita *et al*, 1989). Primer sequences are Goby-cytbF: 5' - CCC ATC AAA CAT TTC TGC -3' and Goby-cytbR: 5'- ACA TAG CCA ACG AAG GC - 3'. PCR conditions for the SSCP reaction were as follows: denaturation at 97°C for 3 min; denaturation at 95°C for 45 s, annealing at 60°C during 45 s, elongation at 72°C for 45 s during 35 cycles. Final elongation took 7 min at 72°C. The $MgCl_2$ concentration was 2 mM. A sample consisting of 5 individuals of *P. lozanoi* was employed as an outgroup; the same primers and conditions were used for amplification and sequencing.

In the SSCP analysis each individual was run several times under identical conditions. A few individuals from each mobility group (depending on the SSCP mobility class frequency) were randomly chosen and their nucleotide sequence was determined on an automated DNA sequencer (LICOR 4200, Westburg). The SSCP was carried out at a temperature of 4°C with a run time of 2 h at 600 V. Bands were visualised by silver staining. PCR conditions for the sequencing reaction were as follows: denaturation at 95°C for 3 min; denaturation at 95°C for 30 s; annealing at 60°C during 15 s; elongation at 70°C for 1 min during 35 cycles. Final elongation was at 70°C during 7 min. Both strands were sequenced. Sequences were analysed with the GeneReadIR DNA system and the AlignIR software (LICOR, Westburg).

Table 2.2.1. *Pomatoschistus minutus*: Geographic location of the sampling sites with abbreviations.

Sampling site	Code	Period	Latitude	Longitude
Trondheim (Norwegian Sea, Central-Norway)	Tro	Sep 2000	63° 24' N	10° 24 E
Bergen (North Sea, Western Norway)	Ber	Sep 1997	60° 23' N	5° 21' E
Tvärminne (Baltic Sea, Finland)	Tva	July 2001	59° 50'N	23° 15'E
Oban (Atlantic Ocean, Scotland)	Ob	Aug 1999	56° 24' N	5° 28' W
Galway (Atlantic Ocean, Ireland)	Ga	Aug 1999	53° 16' N	9° 03'W
Frisian Front (North Sea, The Netherlands)	FrF	Jan 1999	53° 30' N	4 ° E
Texel (Wadden Sea, The Netherlands)	Tex	Dec 1998	53° 00' N	4° 46' E
Doel (Schelde estuary, Belgium)	Do	Oct 1998	51° 19' N	4° 16' E
Stroombank (southern North Sea, Belgium)	Sb	Feb 1997	51° 13'N	2° 52'E
Plymouth (English Channel, UK)	Ply	Nov 1996	50° 22' N	4° 09' W
La Tremblade (Gulf of Biscay, France)	LT	Oct 2000	45° 46' N	1° 08' W
Pérols (Gulf of Lions, France)	Per	Jan 1998	43° 34' N	3° 57' E
Venice (Adriatic Sea, Italy)	Ven	Oct 1999	45° 26' N	12 °19' E

Data analysis

Sequences were aligned in CLUSTALW version 1.7 (Thompson *et al*, 1994). Genetic diversity, measured as haplotype (h) and nucleotide (π) diversity (Nei, 1987) was computed in DNASP version 3.51 (Rozas & Rozas, 1999). Because visual inspection of the sequences revealed a strongly unequal distribution of mutation sites throughout the cytochrome b fragment, the program PUZZLE (Strimmer & von Haeseler, 1996) was used for calculating the parameter α of the gamma distribution. A neighbour-joining haplotype dendrogram (Saitou & Nei, 1987) based on the Tamura-Nei genetic distances was computed in the program MEGA (Kumar *et al*, 2001) The model of Tamura & Nei (1993) takes into account unequal nucleotide frequencies and unequal mutation rates within the fragment. Support for the nodes was assessed with bootstrapping (Felsenstein, 1985) (1000 replicates).

Pair-wise genetic distances between samples were computed in ARLEQUIN version 2.0 (Schneider *et al*, 2000) according to the model of Tamura-Nei (1993). Significance was assessed with permutation tests (1000 replicates). A sequential Bonferroni test was applied to correct

significance levels for multiple testing (Rice, 1989). For assessing patterns of isolation-by-distance a Mantel test (Mantel, 1967) on the geographic versus genetic distance was carried out in GENETIX. In addition, a plot of geographic versus genetic distances was constructed in STATISTICA version 6.0 (STATSOFT, 2001). The pair-wise genetic distances were used as basis for a Multidimensional Scaling Analysis in the program STATISTICA for detecting indications of group structure. Subsequently, samples were grouped and subjected to a hierarchical analysis of variance (AMOVA) (Excoffier *et al*, 1992) in the program ARLEQUIN for assessing whether any significant geographic group structure could be detected.

For studying phylogeographic relationships we constructed a minimum spanning tree, which minimises the connections between the haplotypes, in the program ARLEQUIN. This minimum spanning network was used as basis for a nested design for performing a Nested Clade Analysis (NCA). Nested Clade Analysis (Templeton *et al*, 1995) tests for geographical associations between haplotypes and groups of haplotypes on different hierarchical levels and allows for distinguishing between historical events and contemporary gene flow to explain the observed genetic structure. Significance of the clade distances and the nested clade distances was calculated with permutation tests as implemented in the program GEODIS (Posada *et al*, 2000) (1000 replicates) and interpreted with the inference key according to Templeton (1998). The distance matrix was constructed based on the shortest coastal distance between sites with the electronic atlas ENCARTA (Microsoft, 2001). The parameters of the distribution of pair-wise differences between haplotypes (mismatch distribution) (resp. θ_0, θ_1 and τ) (Rogers & Harpending, 1992) were also calculated in the program ARLEQUIN. The parameter $\tau = \mu T$ with μ the mutation rate and T the time since expansion in generations was used for estimating the time of expansion of the *P. minutus* haplotypes.

RESULTS

Genetic diversity analysis

Twenty-eight haplotypes were recorded among the 228 individuals screened at thirteen sampling sites (Appendix 2.2.1). Global haplotype and nucleotide diversity amounts to 0.627 and 0.0113 respectively. When the Venetian sample is excluded haplotype and nucleotide diversity amount to 0.593 and 0.0037 respectively. The lowest levels of haplotype and nucleotide diversity were

recorded Oban (h = 0.054, π = 0.0002) while the highest values were found in the western Mediterranean Sea (h = 0.811, π = 0.0100). These two samples differed an order of magnitude in both haplotype and nucleotide diversity (Table 2.2.2). No indels were recorded. Forty-four segregating sites were recorded (Appendix 2.2.2), corresponding to 15% polymorphic sites. Thirty-two of the segregating sites were phylogenetically informative, while 12 mutational sites represented singletons. The transition/ transversion ratio was 1.61 and the parameter α of the gamma distribution was 0.23. Base frequencies were T = 0.198, C = 0.186, A = 0.324 and G = 0.291.

Table 2.2.2: Number of fish screened per sampling site (N), number of mtDNA haplotypes (n), number of unique haplotypes (n_h), haplotype diversity (h) and nucleotide diversity (π) with standard deviation between parentheses and % of occurrence of the most common haplotype (MCH). For sample codes see Table 2.2.1.

Sampling site	N	n	n_h	h	π	MCH
Tro	17	3	1	0.228 (0.129)	0.0080 (0.0005)	A (88.24%)
Ber	20	3	0	0.416 (0.116)	0.0015 (0.0005)	A (75.00%)
Tva	11	3	1	0.473 (0.026)	0.0018 (0.0007)	A (72.73%)
Ob	37	2	1	0.054 (0.050)	0.0002 (0.0002)	A (97.28%)
Ga	4	1	0	0	0	A (100.00%)
FrF	15	5	2	0.771 (0.072)	0.0043 (0.0006)	F (40.00%)
Tex	24	6	3	0.659 (0.085)	0.0037 (0.0009)	F (54.16%)
Do	26	3	0	0.628 (0.065)	0.0031 (0.0004)	F (53.85%)
Sb	20	3	0	0.532 (0.100)	0.0024 (0.0005)	F (65.00%)
Ply	20	3	2	0.195 (0.0131)	0.0024 (0.0015)	A (90.00%)
LT	2	1	0	0	0	A (100.00%)
Per	22	10	8	0.844 (0.062)	0.0100 (0.0011)	A (36.36%)
Ven	10	5	5	0.822 (0.097)	0.0096 (0.0016)	N (40.00%)

Haplotype distribution

Haplotype A is the most widespread, being present in appreciable frequencies in all samples, with the exception of the Venetian sample, where it is absent (Table 2.2.2). The sample from Pérols is the most diverse, with ten haplotypes recorded among 22 individuals. Eight haplotypes are unique

to this sample and only two are shared with the Atlantic samples (resp. A and K). All Venetian haplotypes are exclusive: none occurred in other samples of Pomatoschistus minutus and vice versa. Haplotypes F and G occur commonly in the four samples of P. minutus collected in the southern North Sea (Doel, Stroombank, Texel, Frisian Front), but were not recorded in the samples north of the Frisian Front or south of the Belgian coast (Appendix 2.2.1). Only one haplotype was found in the samples from the Gulf of Biscay and Galway, but from these sites only 2 respectively 4 individuals were available. The least diverse samples containing a substantial number of individuals were Oban and Plymouth, where haplotype A occurred in at least 90% of the fish screened (Table 2.2.2).

The haplotype dendrogram clearly reveals (1) the complete isolation of the Venetian haplotypes, (2) the western Mediterranean as a separate cluster from the Atlantic *P. minutus*, (3) a shallow phylogeographic structure within the Atlantic basin and (4) a higher level of divergence between the western Mediterranean haplotypes than between Atlantic haplotypes. Bootstrap values between the Atlantic haplotypes were very low, and usually lower than 30% (not shown). Furthermore, the dendrogram suggests that the Venetian lineage is even older than the split between *P. minutus* and *P. lozanoi* (Fig. 2.2.2). Pair-wise genetic distances (not shown) between sequences varied between 0.007 to 0.018 for the Atlantic *P. minutus* haplotypes, while genetic distance between *P. lozanoi* and *P. minutus* ranged from 0.087 to 0.095. The genetic distance between the Adriatic *P. minutus* and the others was slightly higher, ranging from 0.104 to 0.117.

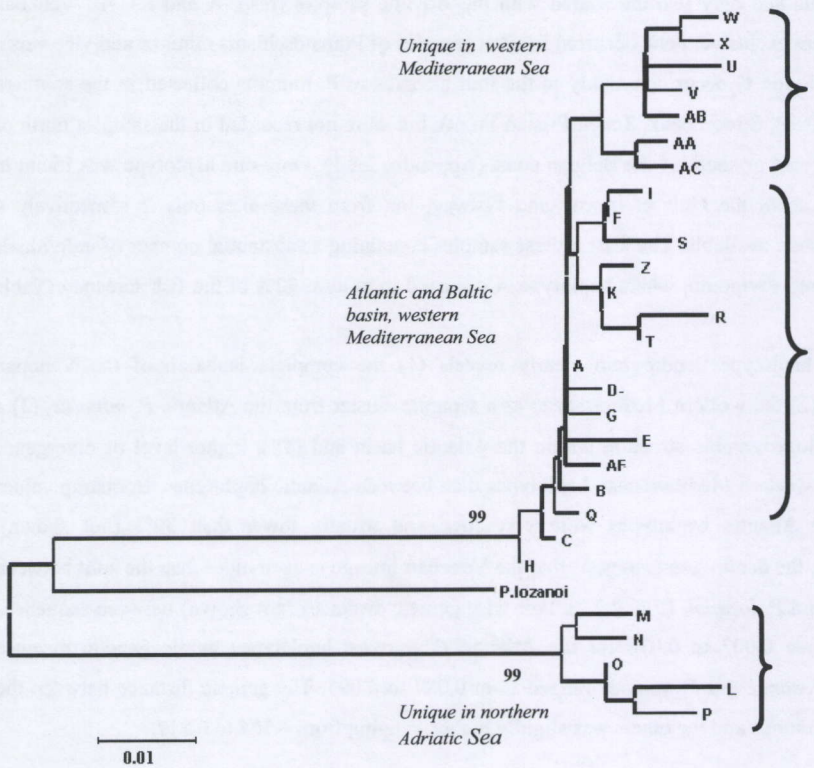

Figure 2.2.2. *Pomatoschistus minutus*: Neighbour-joining haplotype dendrogram based on Tamura-Nei genetic distances. Only bootstrap values higher than 75% are presented.

Genetic diversity and latitude

A linear regression of both haplotype and nucleotide diversity versus latitude yielded a significantly negative correlation (resp. $R^2 = 0.39$, $P = 0.040$ and $R^2 = 0.69$, $P = 0.001$) (Fig. 2.2.3). The samples from Galway and La Tremblade were not taken into account for the regression analysis due to the low number of individuals screened. Outliers in the regression are the British samples (Oban and

Plymouth), showing a very low level of haplotype diversity. This is due to the almost exclusive occurrence of haplotype A, occurring in more than 90% of the samples screened (Table 2.2.2).

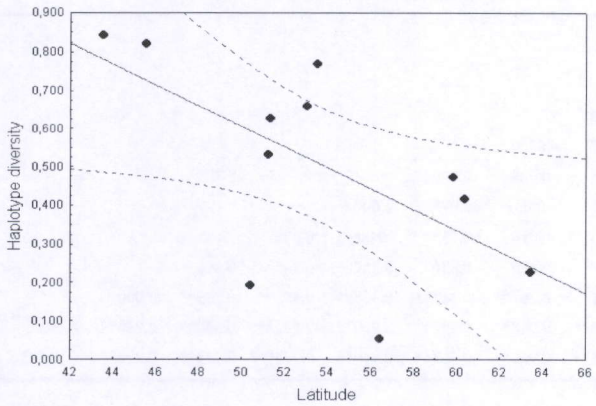

Figure 2.2.3. *Pomatoschistus minutus:* Regression of the haplotype diversity plotted against latitude. The dashed line represents the 95% confidence interval. $R^2 = 0.39$, $P = 0.040$.

Population differentiation of *Pomatoschistus minutus* throughout its distributional range

The values of the pair-wise genetic distances are presented in Table 2.2.3 and a sample of *Pomatoschistus lozanoi* is added for comparison. The samples from Galway and La Tremblade were not included because of the low sample size (respectively only four and two fish screened). It is clear that the largest values of pair-wise distances are found between the Venetian sample and all the others. Table 2.2.3 shows furthermore that this differentiation is comparable to the genetic distance between *Pomatoschistus minutus* and the sample of *P. lozanoi*. The sample from Pérols (Gulf of Lions, western Mediterranean Sea) is also significantly differentiated from the Atlantic *P. minutus*. Pair-wise genetic distances within the northeastern Atlantic Ocean and the Baltic Sea were generally not significant.

Table 2.2.3: Pair-wise genetic distance (Tamura-Nei, 1993) for all samples of *Pomatoschistus minutus*. Values indicated with an * or ** are significant at the 0.05 and 0.01 level respectively after sequential Bonferroni correction. A population of *P. lozanoi* (*Pl*) is added for a comparison. For sample abbreviations see Table 2.2.1.

	Tro	**Ber**	**Ob**	**Tva**	**FrF**	**Tex**	**Do**	**Sb**	**Ply**	**Per**	**Ven**	***Pl***
Tro	0.000											
Ber	0.021	0.000										
Ob	0.027	0.161**	0.000									
Tva	0.063	0.101	0.176	0.000								
FrF	0.273	0.279	0.423	0.208	0.000							
Tex	0.071	0.106	0.133	0.039	0.028	0.000						
Do	0.337	0.341**	0.455	0.301	-0.039	0.078	0.000					
Sb	0.471	0.451**	0.614	0.415	0.019	0.135	-0.007	0.000				
Ply	0.008	0.062	0.044	0.002	0.189	0.026	0.257	0.345	0.000			
Per	0.234**	0.252**	0.342**	0.185	0.267**	0.243**	0.338**	0.353**	0.229**	0.000		
Ven	0.965**	0.964**	0.982**	0.953**	0.948**	0.956**	0.960**	0.962**	0.960**	0.920**	0.000	
Pl.	0.984**	0.979**	0.993**	0.976**	0.960**	0.963**	0.969**	0.974**	0.973**	0.920**	0.934**	0.000

Because of the large differentiation between the *P. minutus* from the northern Adriatic Sea and the other samples (Fig. 2.2.1, Table 2.2.3), the Venetian sand gobies were left out for further population genetic and phylogeographic analysis for avoiding distortion of the results.

Isolation-by-distance

A Mantel test for all samples (except for Galway and La Tremblade) showed a significant correlation between geographic and genetic distances ($r = 0.76$, $P = 0.0062$), but when the Venetian sample was excluded, no significant effect was observed anymore ($r = 0.02$, $P = 0.249$). Yet, a clear pattern of isolation-by-distance is observed when geographic distance is plotted against genetic distance. (Fig.2.2.4).

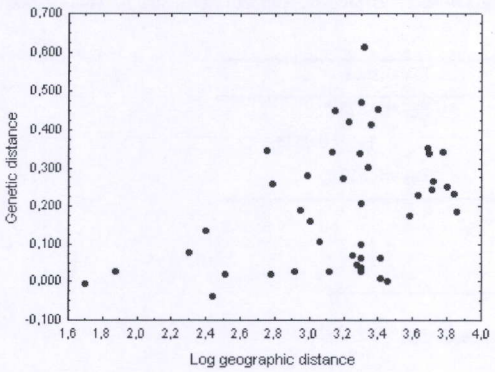

Figure 2.2.4 *Pomatoschistus minutus*: Plot of log-transformed geographical distances against genetic distances for all samples. The Venetian sample is excluded.

Genetic structure of the sand goby within the Atlantic and western Mediterranean basins

A Multidimensional Scaling Analysis was performed on all samples except for Venice for assessing whether any indication for grouping structure could be found. Figure 2.2.5 shows that the samples from the southern North Sea (dominated by haplotype F) are clustering together, while the samples from Norway, Scotland and the English Channel (haplotype A-dominated) form also a distinct group, indicating at least some degree of geographic structuring within the Atlantic sand gobies. In order to ascertain if the structure was significant, we carried out an AMOVA analysis on the Atlantic samples. Samples were divided into two groups: 1) the southern North Sea (Doel, Stroombank, Frisian Front and Texel) and 2) all the others (Baltic, Sea, Oban, Bergen and Trondheim). The AMOVA showed that most variation was found within populations (68 %), while 27 % was due to variation between the two geographic groups (southern North Sea - others). Four percent was explained by variation among samples. All values were highly significant. (Table 2.2.4)

Table 2.2.4. Hierarchical analysis of variance (AMOVA) based on mtDNA haplotypes scored in the Atlantic samples of *Pomatoschistus minutus*. Group 1 = Southern North Sea; Group 2 = Baltic Sea, Northern North Sea, Atlantic Ocean.

Source of variation	% of variation	F-statistics
Among groups	27.83	$F_{CT} = 0.278$ *
Among populations within groups	3.88	$F_{SC} = 0.054$*
Within populations	68.29	$F_{ST} = 0.317$ *

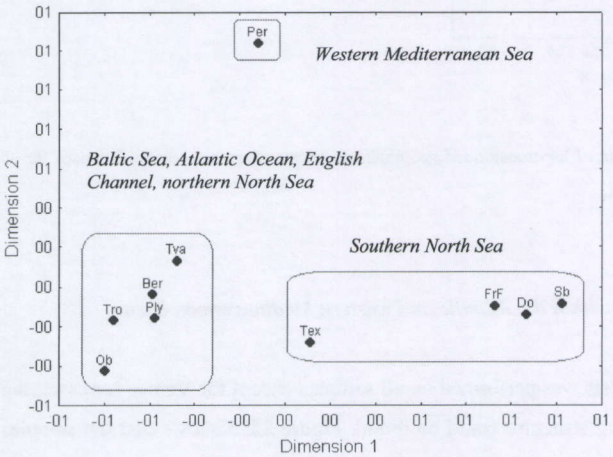

Figure 2.2.5. *Pomatoschistus minutus:* first and second dimension of the Multidimensional Scaling Analysis. The Venetian sample is excluded for avoiding distortion. Stress value = 0.000. Sample abbreviations are listed in Table 2.2.1.

Phylogeographic analysis

Nested Clade Analysis

Intraspecific relationships between haplotypes are often better represented by a network than by a bifurcating tree because they are not hierarchical: a natural population consists of a combination of

ancestral haplotypes and new variants (Posada & Crandall, 2001). The minimum spanning tree (Fig. 2.2.6) shows that the haplotypes of *Pomatoschistus minutus* are grouped in two geographically distinct clusters: Clade III-1 comprising the unique western Mediterranean haplotypes and Clade III-2, which contains the Atlantic haplotypes. Clade III-1 and III-2 are connected through haplotype K, which is present in both basins. The topology of the clusters is clearly different: the Atlantic clade shows a star-like pattern with one central and common haplotype A and a number of rare variants radiating, which are generally separated by only one mutational step from haplotype A. The Mediterranean cluster reveals more differences between the various haplotypes and no clear star-like pattern is observed. Permutation analysis of clade distances versus nested clade distances revealed that the null hypothesis of no geographical association of haplotypes could be rejected at several levels in the nested clade analysis. In the lowest level (1-step) clade containing haplotype A (clade 1-11), restricted gene flow and isolation-by-distance was suggested. At the 2-step level a contiguous range expansion was suggested for clade 2-5 (containing haplotype F), which is the dominating haplotype in the Southern Bight. Restricted gene flow and isolation by distance was suggested for Clade 2-4. Due to the uncertain tip/interior status of clades III-1 and III-2, no analysis of the total cladogram could be performed.

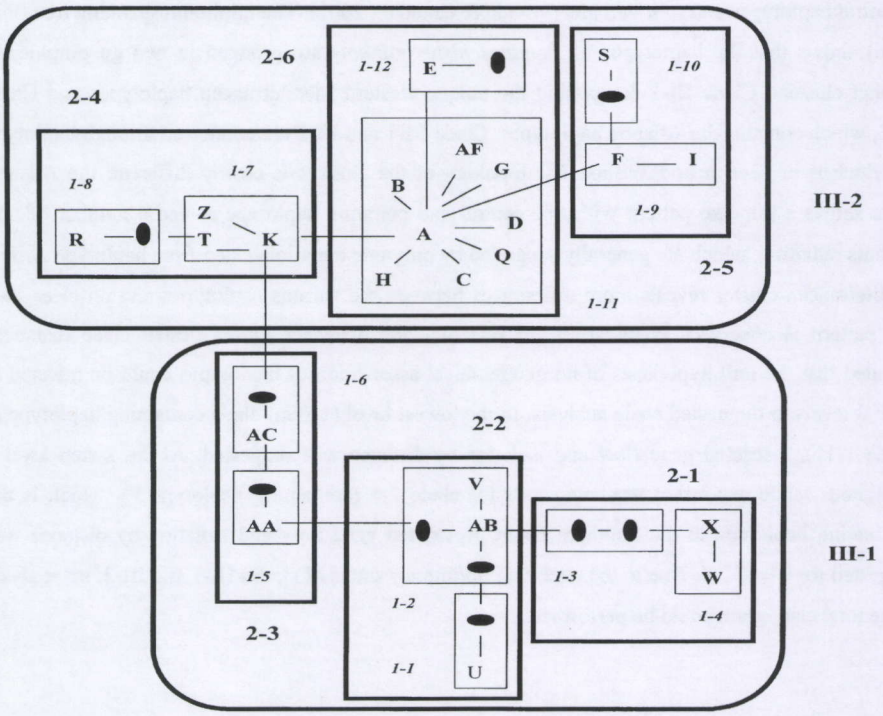

Figure 2.2.6. *Pomatoschistus minutus*: nested design following Templeton *et al* (1992). Black ovals represent missing haplotypes. Each branch represents one mutational step and branch length is not proportional to the distance between the haplotypes.

Table 2.2.5. *Pomatoschistus minutus*: Phylogeographical inferences from the Nested Clade Analysis (Templeton, 1998). Only clades where the null hypothesis could be rejected are represented. For the nested design, see Fig. 2.2.6.

Clade	dominating haplotype / geographical distribution	Inference
1-11	A/ all sites	Restricted gene flow and isolation-by-distance
2-4	K/ western Mediterranean Sea, Atlantic Ocean	Restricted gene flow and isolation-by-distance
2-5	F/ Southern Bight	Contiguous range expansion

Mismatch distribution

As the star-like phylogeny of the Atlantic clade pointed to a population expansion, the mismatch distribution was calculated for assessing the time of expansion of the Atlantic haplotypes. Tests of the goodness-of-fit revealed that the model of a sudden population expansion could not be rejected (P = 0.000). The values of the parameters τ, Θ_0 and Θ_1 were 0.731, 0.088 and 1669 respectively. Tajima's D-statistic amounted to - 1.83 and was significantly negative (P = 0.019), further supporting the model of a sudden expansion. Assuming a mutation rate of 2% per Ma, this corresponds to time of expansion of 130 ka BP.

DISCUSSION

The results point to (1) the complete isolation of the northern Adriatic sand gobies, (2) a significant differentiation between sand gobies of the western Mediterranean and the Atlantic Ocean and (3) limited genetic differentiation between sand gobies within the Atlantic and Baltic basins with a weak pattern of isolation-by-distance, but a distinction in two groups.

The Mediterranean Sea

Three striking patterns are recorded for *Pomatoschistus minutus* in the Mediterranean basin: (1) the complete isolation of the northern Adriatic *P. minutus,* (2) the high level of endemism of the Mediterranean haplotypes, and (3) the presence of the Atlantic haplotype A in an appreciable frequency.

A cryptic species of the sand goby in the northern Adriatic Sea

Analysis based on the cytochrome b results strongly supports the findings in Paper I: the genetic distance as observed with allozymes and sequences is similar to the distance between *P. minutus* and *Pomatoschistus lozanoi.* When recalculating our distances using the K2P model we obtained values ranging from 0.092 to 0.113 for the difference between *P. minutus* and *P. lozanoi*, and a

distance from 0.122 to 0.147 between Adriatic and other *P. minutus*. This falls within the range reported for interspecific differences by Johns & Avise (1998). Clearly, the Adriatic population must have been subject to a long-lasting isolation. This contrasts with the suggestion of Stefanni & Thorley (2003), who date the split between the Adriatic and other Mediterranean sand gobies between 5 and 10 ka BP based on D-loop sequences. They suggest that sand gobies in the Mediterranean Sea migrated northwards upon rising temperatures after the last glacial period and became trapped in the cul-de-sac of the Adriatic Sea. The present isolation of the northern Adriatic *P. minutus* would be maintained by a temperature barrier in the southern Adriatic, where summer sea surface temperature may be as high as 21°C. However, an alternative hypothesis may be linked to the hydrology of the Adriatic basin with a large gyre in the south, which partially isolates the northern Adriatic Sea from the Mediterranean Sea (Artegiani *et al*, 1993). This has been proposed as an isolating mechanism for the European anchovy (Magoulas *et al*, 1996).

Sequence analysis of 12S and 16S (Huyse, 2000) confirms the genuine reproductive isolation of the Venetian *P. minutus*. An extensive morphometric study did not reveal sufficient differentiation for discriminating between the Venetian and other *P. minutus* on morphological grounds (Stefanni, 2000a). However, lack of congruence between results from morphological and genetic markers is often observed (Chow & Kishino, 1995; Turan *et al*, 1998). Fish are subject to phenotypic plasticity, suggesting that morphological criteria might not be appropriate for delineating species' boundaries. Morphologically similar populations often turn out to be genetically distinct, representing cryptic species (Gilles *et al*, 2000, Bucciarelli *et al,* 2002). Huyse (2000) suggest a more ancient split of the Adriatic *Pomatoschistus minutus* population than the speciation event within the *P. minutus* complex. This is also supported by our haplotype dendrogram. Allozyme results show a different picture, and suggest that the split between *P. minutus* and *P. lozanoi* is oldest. However, the resolution of allozymes for timing speciation events is limited (Avise, 1994). Assuming a molecular clock between 1% (slow) and 2 % (fast) per 10^6 years (Ma) for the cytochrome b, this would mean that the Venetian sand gobies, which differ 9.19%, from the other *P. minutus,* must have been isolated between 4.5 and 9 Ma BP. It has been suggested that the Messinian salinity crisis (Hsü *et al*, 1977), which occurred between 5.96 and 5.33 Ma BP, played a major role in the speciation of Mediterranean gobies (Miller, 1990). During this period, the Mediterranean basin was completely isolated from the Atlantic Ocean and desiccated, resulting in the formation of a series of hypo- and hypersaline lakes, and the extinction of most of the existing marine fauna. *P. minutus* is adapted to brackish-water conditions and hence might have

been able to survive in river systems in the northern Adriatic basin. However, this time frame conflicts with the view of Huyse (2000), who suggests a more recent origin of the Adriatic *P. minutus,* of about 1.73 -1.13 Ma BP, based on the 12S and 16S mitochondrial sequences. In this interpretation sea-level changes during glaciations might have caused isolation of the Adriatic basin and induced allopatric speciation of a relict population of proto-*P. minutus*. The small fragment of the cytochrome b (283 bp), which may have been hypervariable and has a limited resolution for addressing phylogenetic issues, might have biased our estimation. Penzo *et al* (1998) found no concordance between the timing of speciation of Mediterranean freshwater gobies and the Messinian salinity crisis either. In order to correlate these events an (unlikely) almost fivefold faster rate of evolution for the goby mtDNA compared to other fish species had to be invoked. Nevertheless, the estimates of allozymes, cytochrome b, 12S and 16S show that the split between the Adriatic and the other sand gobies has probably a much older origin than the 5 to 10 ka suggested by Stefanni & Thorley (2003). Screening of additional loci or a larger fragment would probably shed more light on the evolutionary history and speciation processes within the Mediterranean sand gobies.

The western Mediterranean Sea

The origin of the Atlanto-Mediterranean gobies lies within the Mediterranean basin, where the oldest known fossil goby remains were found (Simonovic, 1999). It is thought that ancestors of the present goby fauna migrated into the Atlantic Ocean and evolved in allopatry during the Messinian salinity crisis. After reflooding of the Mediterranean basin at 5.3 Ma BP they re-invaded the Mediterranean basin through the Strait of Gibraltar. Huyse (2000) suggests an Atlantic origin for the three species of the *P. minutus* complex (Webb, 1980), which consists of *Pomatoschistus minutus, P. lozanoi* (de Buen, 1923) and *P. norvegicus* (Collett, 1902) triggered by the Pleistocene glacials and interglacials. However, this implies two independent and almost simultaneous speciation events at two geographically separate areas from the same proto-*P. minutus* ancestor: one in the Adriatic Sea and one in the northeastern Atlantic Ocean. An alternative, more parsimonious hypothesis would be that speciation within the *P. minutus* complex took place in the Mediterranean Sea. A population of proto-*P. minutus* might have become isolated in the Adriatic Sea while another population of this ancestor might have given rise to the three species of the *P. minutus* complex elsewhere in the Mediterranean Sea. This may have been during the Messinian or Pleistocene sea-

level drops, when populations of various basins got isolated. When the connection with the Atlantic Ocean was re-established, populations of the three *P. minutus* - complex species may have dispersed into the Atlantic Ocean. Although *P. lozanoi* has never been recorded from the Mediterranean Sea, this species has long been considered a subspecies of *P. minutus* and thus its presence may have been overlooked elsewhere (Hamerlynck, 1990). Alternatively, the stenotopic *P. lozanoi* might have died out in the Mediterranean Sea because of unfavourable ecological conditions. *P. norvegicus* on the contrary, has been recorded at several locations in the Mediterranean Sea (Stefanni, 2000b and references herein).

Support for this hypothesis is provided by the haplotype distribution and diversity of the sand goby. The Minimum Spanning Tree shows the endemic Mediterranean haplotypes as a separate cluster. A genetic architecture characterised by a high nucleotide and haplotype diversity may be attributed to secondary contact of different mitochondrial lineages, which evolved in allopatry (Bowen & Grant, 1998). Magoulas *et al* (1996) suggested such a scenario is for explaining the haplotype distribution of the European anchovy.

Hence, we propose that the most common haplotype A and its descendants originated more recently in the Atlantic from a Mediterranean ancestor, invaded the Mediterranean Sea and came into secondary contact with the resident *P. minutus.* Alvarado-Bremer *et al* (1995) found a similar pattern of differentiation between Atlantic and Mediterranean populations of the swordfish *Xiphias gladius*, suggesting both historical separation and ongoing gene flow.

Contemporary gene flow between Atlantic and Mediterranean sand gobies

Because of the physical oceanography at the Atlanto-Mediterranean boundary (Krijgsman, 2002), any gene flow at present is likely to be unidirectional from the Atlantic into the Mediterranean. The pattern of isolation-by-distance for haplotype A at the 1-step level in the NCA suggests at least some ongoing gene flow across the Almeria-Oran front. Yet, a significant degree of differentiation between Atlantic and western Mediterranean *Pomatoschistus minutus* is observed. These findings contrast with other studies on the sand goby, where limited or no differentiation between western Mediterranean and North Sea samples of *P. minutus* was found (Stefanni 2000a; Huyse 2000). Stefanni (2000a) employed allozymes, as is the case in Paper I, which in general have a lower resolution than the mtDNA for detecting population structure. Huyse (2000) did not record any differentiation between gobies from the western Mediterranean Sea and the Belgian coast.

However, this study was based on the 12S and 16S loci, and thus might have failed to detect recent population divergence because of the choice of a rather conservative marker.

Phylogeographic patterns of the sand goby in the northeastern Atlantic Ocean

The mismatch analysis, the negative value of Tajima´s D-statistic and the star-like pattern in the minimum spanning tree point to a population expansion of *Pomatoschistus minutus* in the Atlantic Ocean. The value for τ suggests that this would have happened about 130 ka BP. This coincides with the onset of the Eemian, the interglacial before the last glaciation event, which lasted about 20 ka (Lowe & Walker, 1997). *P. minutus* must have experienced a similar range expansion into northern areas as well, before it was pushed back again by the last glacial event, the Weichselian (110-10 ka BP).

Loss of genetic variation at higher latitudes is common in populations inhabiting formerly glaciated areas, and is usually attributed to founder events during range expansion following deglaciation (Hewitt, 2000). According to this hypothesis the low level of diversity in the British samples (Plymouth, Galway and Oban) is probably best explained by a founder event during postglacial range expansion by a small and/or genetically homogeneous population of *P. minutus* dominated by haplotype A, as suggested by the 2-step level in the NCA. Alternatively, selection on mtDNA haplotypes (Mishmar *et al*, 2003) or lineage sorting (Gabrielsen *et al*, 2002) may yield a similar pattern. A similar loss of variation in British compared to continental populations has been reported for the related common goby, *Pomatoschistus microps* (Paper III).

While haplotype A is common in the *Pomatoschistus minutus* from the southern North Sea as well, these samples are dominated by haplotype F. Considering that haplotype F seems to be unique to this area, a glacial refugium for *P. minutus* north of the English Channel may explain this distribution. This is supported by the NCA, suggesting a contiguous range expansion of this clade in the southern North Sea. Verspoor *et al* (1999) proposed a refugium for salmon in the southern North Sea. García-Marin *et al* (1999) suggested a glacial refugium for brown trout in the drainage systems of the major rivers which flowed through the English Channel during periods of lowered sea level (Gibbard, 1988). *P. minutus* is adapted to the estuarine environment and thus isolated populations may have survived in brackish water. In this view, the Southern Bight would constitute

a zone of secondary contact between (1) haplotype A and its descendants, expanding northward from a southern refugium, and (2) a resident population dominated by haplotype F.

The Baltic Sea

Studies on salmon and trout revealed phylogenetically different lineages within the Baltic Sea, suggesting distinct postglacial recolonisation events from the adjacent North Sea as well as from refugia in glacial lakes east of the Baltic Sea (Osinov & Bernatchez, 1996; Koljonen et al, 1999). Although the sample of *Pomatoschistus minutus* was collected in the eastern Baltic Sea, the dominating haplotype was the most common Atlantic haplotype (A), suggesting an invasion from the North Sea by haplotype A via a contiguous range expansion. The low degree of haplotype diversity compared with the North Sea samples might be attributed to founder events similar to the samples from the British Isles. No significant differentiation from the Atlantic *P. minutus* is recorded. A similar lack of differentiation between Baltic and Atlantic populations was recorded for flounder (Borsa *et al*, 1997a). However, water exchange with the North Sea is severely restricted. As such, it is not unlikely to assume limited gene flow between Baltic and North Sea in species depending on a planktonic larval stage for dispersal. Considering the fact that invasion of the Baltic Sea by *P. minutus* is less than 8,000 years old, populations might still not be at equilibrium. Alternatively, the resolution of mtDNA might not be sufficient for detecting processes of ongoing and recent population divergence. For example, microsatellite analysis showed clear genetic differences between Atlantic and Baltic cod (Nielsen *et al*, 2001) and a hybrid zone in between (Nielsen *et al*, 2003). Microsatellite screening of sand goby populations in the Southern Bight yielded evidence for distinct breeding units on a small scale (Paper 5). Thus, for more final conclusions regarding ongoing gene flow of *P. minutus* between the Atlantic Ocean and the Baltic Sea, the use of more sensitive marker is warranted.

CONCLUSIONS

The genetic structure of the sand goby is best explained by a combination of recurrent and historic factors. Due to its pelagic larval stage the species has a high potential for dispersal via oceanic currents. This is reflected in the weak genetic differentiation and the pattern of isolation-by-distance

within the Atlantic and the Baltic basins. A glacial refugium for *Pomatoschistus minutus* in the southern North Sea is proposed as the distribution of haplotypes in the area suggests a secondary contact after a period of isolation. Alternatively, we cannot exclude the effect of lineage sorting. The occurrence of the most common Atlantic haplotype in the western Mediterranean Sea in an appreciable frequency indicates ongoing gene flow across the Almería-Oran front. Yet, a high proportion of unique haplotypes is found in the population from the western Mediterranean Sea. This points to an historical isolation followed by secondary contact between sand gobies invading the Mediterranean Sea from the Atlantic Ocean and resident *P. minutus*.

The large genetic distance between the Venetian *P. minutus* and all the others supports the hypothesis of allopatric speciation in the northern Adriatic Sea. This may be linked to isolation of the Adriatic basin during the Pleistocene or the Pliocene. Regarding the timing of this speciation event, results for the various genetic markers conflict. The screening of additional genes would be warranted for more final conclusions regarding speciation processes of Mediterranean sand gobies.

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APPENDICES

Appendix 2.2.1: Haplotype distribution *of Pomatoschistus minutus*. For the sample abbreviations see Table 2.2.1.

H	Tro	Ber	Tva	Ob	Ga	FrF	Tex	Do	Sb	Ply	LT	Per	Ven	Total
A	15	15	8	36	4	3	13	6	5	18	2	8		133
B	1	4												5
C		1												1
D	1													1
E							1							1
F						6	6	14	13					39
G						4	2	6	2					14
H												1		1
I						1								1
K			2			1						1		4
L													1	1
M													1	1
N													4	4
O													2	2
P													2	2
Q			1											1
R										1				1
S										1				1
T							1							1
U												1		1
V												1		1
W												2		2
X												2		2
Z							1							1
AA												1		1
AB												4		4
AC												1		1
AF				1										1
Total	17	20	11	37	4	15	24	26	20	20	2	22	10	228

Appendix 2.2.2. *Pomatoschistus minutus*: partial cytochrome b sequences of the various haplotypes with EMBL accession numbers (AN). Only variable sites and their positions are represented. H refers to the various haplotypes. Haplotype A is employed as reference sample.

AN	H	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2
		1 1 1 2 2 3 3 4 5 6 6 7 8 9 9 1 2 3 3 3 6 6 8 8 0 0 1 2 2 3 4 4 4 4 5 5 5 5 6 6 6 7 8 8
		3 6 9 2 9 4 5 6 8 0 4 6 8 4 7 1 4 3 6 9 3 9 4 7 2 5 1 0 3 5 1 4 6 7 0 3 6 9 2 5 8 4 0 3
AJ555096	A	G C G C A T C T A C A G T T A T T C A C C T C T C C A C T A C G T G T A A A C A A A A C
AJ555097	B	. . . . . A . . . . .
AJ555098	C	A . . . . .
AJ555099	D	. . . G . . . . .
AJ555100	E	. T . . . . . C . . . . .
AJ555101	F	. . . . . G . . . . .
AJ550102	G	. . . . . C . . . . .
AJ555103	H	. . . . . G . . . . .
AJ555118	I	. . . . . G . . C . . . . .
AJ555104	K	. . . A . . . . .
AJ555105	L	A G T C . C . . G . G A A C . G C G . . T . T C . A G . C . T . . T C . . . . . G G G T
AJ555106	M	A G T C . C . . G . G . A C . . C G G . T C T C . A G . C . T . . C C . . . . . G G G T
AJ555107	N	A G T C . C . . G . G . A C . . C G G . T . T C . A G . C . T . . T C . . G . . G G G T
AJ555108	O	A G T C . C . . G . G . A C . G C G . . T . T C . A G . C . T . . T C . . . . . G G G T
AJ555109	P	A G T C . C . . G . G . A C . G C G G T T . T C . A G . C . T . . T C . . . . . G G G T
AJ555110	Q	. . . . . C . . . . .
AJ555111	R	. . A A . . . . . C . . . . .
AJ555112	S	. . . . . G G . . . . . G . . . . .
AJ555113	T	. . A A . . . . .
AJ555114	U	. . . . . G . C . . G G . .
AJ555115	V	. . . . . A . . . . . G . C . . . . .
AJ555116	W	. . . . . T . . . . . G . C . . . . . T
AJ555117	X	. . . . . C . . . . . G . C . . . . . T
AJ555119	Z	. . . A . . . . . T . . . . .
AJ555120	AA	. . . . . A . . A . . . . . T . . C . . . . .
AJ555121	AB	. . . . . A . . . . . G . C . . . . .
AJ555122	AC	. . . A . . A . . A . . . . .
AJ555123	AF	. . . . . A . . . . .

CHAPTER III

GENETIC STRUCTURE AND PHYLOGEOGRAPHY OF THE COMMON GOBY, *POMATOSCHISTUS* MICROPS (KRØYER, 1938), THROUGHOUT ITS DISTRIBUTIONAL RANGE

PAPER III

Phylogeographic patterns of the common goby, *Pomatoschistus microps*, a small demersal fish along the northeastern Atlantic coasts and in the Mediterranean Sea

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ABSTRACT

The phylogeographic patterns of a small marine fish, the common goby, *Pomatoschistus microps* (Krøyer 1838), were assessed along the northeastern Atlantic coasts. A combination of two genetic markers was employed: cellulose acetate allozyme electrophoresis (CAGE) and sequence analysis of a 289 bp fragment of the mitochondrial locus cytochrome b. Twelve coastal sites were sampled, ranging from central Norway and the British Isles to Portugal and the Western Mediterranean Sea.

Both markers were congruent in revealing significant differences between most populations and a pattern of isolation-by-distance along the European coasts. Phylogeographic analyses yielded a shallow branching structure with four groups; three were confined to the Atlantic basin and showed a starlike pattern, which may be due to either a relatively recent (postglacial) population expansion after a bottleneck or a founder event. The fourth group contained a central haplotype occurring at the edges of the species' distribution (resp. the Norwegian and Mediterranean Sea) accompanied by a few rarer variants, which were restricted to the Mediterranean Sea. A genetic break was observed around the British Isles, with distinct haplotypes dominating at either side of the English Channel. Gene flow analysis suggested that recolonisation of the North Sea and the coasts of western Scotland and Ireland may have taken place from a glacial refugium the Southern Bight of the North Sea. Alternatively, the present distribution of the haplotypes, which corresponds to a Lusitanian/Boreal distribution, may be influenced by temperature-dependent selection. A significantly negative correlation between the degree of genetic diversity and latitude was recorded both for mtDNA and allozymes in the Atlantic basin. Historical factors such as founder effects during recolonisation of formerly glaciated areas are probably responsible for the loss of genetic variation at higher latitudes in the northeastern Atlantic Ocean

INTRODUCTION

While allozyme-based surveys allow for drawing conclusions about the degree of intra- and interspecific genetic differentiation, the interpretation of how and when this differentiation arose is necessarily limited due to the low historical resolution of these markers. This has changed with the application of mitochondrial DNA analysis (Avise *et al*, 1987), which is particularly well suited for addressing historical issues because of its maternal inheritance, lack of recombination and faster rate of evolution as compared to the coding nuclear DNA. Thus, there has been an exponential increase of phylogeographic studies based on mtDNA analysis recent years (Avise, 2000 and references herein). However, most studies so far have focused on terrestrial and freshwater species. (e.g. Merilä *et al,* 1997; Clausing *et al,* 2000; Hewitt, 2000; Volckaert *et al,* 2002). This is not surprising, as the generally high degree of genetic structuring in these groups makes it relatively straightforward to correlate patterns of genetic divergence with past paleoclimatological and vicariance events. For example, the Pleistocene glaciations have left a distinct genetic imprint on

the structure of many freshwater and terrestrial species, which can be interpreted as a postglacial range expansion from one or multiple ice-free refugia in the south (Bernatchez & Wilson, 1998; Taberlet *et al*, 1998; Hewitt, 2000). A rich literature on phylogeographic patterns of anadromous fish such as salmon and brown trout is also available (e.g. Garciá-Marín *et al*, 1999; Verspoor *et al*, 1999; Consuegra *et al*, 2002).

However, phylogeographic patterns in genuine marine species have been studied to a much lesser degree. Compared to the formidable barriers for dispersal posed to terrestrial and freshwater species, the marine environment is much more homogeneous. Hence, in general marine fish show a low degree of genetic differentiation (see Ward *et al*, 1994, for a review). This makes it more difficult to detect historical and ongoing processes of population divergence. A number of studies have been dealing with oceanic species for resolving biogeographical issues (Palumbi, 1996; Bowen & Grant, 1997; Lessios *et al*, 1999). Other studies have targeted reef fishes (Muss *et al*, 2001; Planes & Fauvelot, 2002; Rocha *et al*, 2002). While some phylogeographical patterns of fish and invertebrates along the Pacific American coast have been elucidated (Bernardi *et al*, 1993; Stepien *et al*, 2001), the evolutionary history of the northeastern Atlantic marine fauna remains poorly documented (but see Wilke & Davis, 2000; Wilke & Pfenniger, 2002). Exceptions are commercially exploited fish species such as mackerel (Nesbø *et al,* 1999), sea bass (García de Leon *et al*, 1997), flounder (Borsa *et al*, 1997a) and cod (Nielsen *et al*, 2003). Yet, phylogeographic patterns are less easily resolved in these species due to high levels of gene flow. Highly vagile fish species often show no mtDNA heterogeneity on a scale of hundreds of kilometers, which necessitates the use of highly variable markers. Due to their extremely high variability and mutation rate, these markers are often less appropriate for inferring historical processes. Thus, for elucidating phylogeographic patterns of the marine fauna along the European coasts we need to employ a model organism showing lower levels of gene flow.

The common goby, *Pomatoschistus microps*, is one of the most abundant estuarine and coastal fish species along the European coasts (Healey, 1972). Its range of distribution extends along the Atlantic coast from central Norway (Trondheimsfjorden), around the British and Irish coasts to Morocco and the Baltic Sea (Miller, 1986). In the Mediterranean Sea its occurrence is limited to lagoons and estuaries in the Gulf of Lions and in Corsica (Bouchereau & Guelorget, 1997). Adults are considered poor swimmers because the pelvic fins are fused in a suction disc (Miller, 1986), suggesting that any large-scale dispersal is likely to depend on the pelagic larval stage, which lasts between six and nine weeks (Jones & Miller, 1966). Mediterranean *P. microps*

complete their entire life cycle including reproduction in estuaries and lagoons (Bouchereau & Guelorget, 1997), while Atlantic common gobies perform spawning and thermal migrations (Jones & Miller, 1966). Adult *P. microps* are rarely encountered offshore and their habitat is limited to very shallow tidal pools and estuaries. Thus, despite its pelagic larval phase, *P. microps* is probably limited in its dispersal due to ecological constraints, and might reveal a clear phylogeographic structure. Hence, in this study we use the common goby as a model for gaining a better knowledge of the evolutionary patterns of marine fish along the European coasts. More specifically we aim to test the following hypotheses:

(1) The Pleistocene glaciations did not only influence freshwater and terrestrial organisms, marine species have also been heavily affected. Ice-covered areas to the north were unsuitable habitats, forcing a southward migration. Thus, in the northeastern Atlantic Ocean we expect the genetic imprint of a postglacial northward range expansion from a southern source population, probably along the Iberian peninsula. We predict a loss of genetic diversity in *P. microps* populations at higher latitudes (Hewitt, 1996). However, the effect of natural selection might also be responsible for the loss of genetic variation commonly observed at higher latitudes (Mishmar *et al*, 2003). Hence, we employ a combination of two independent markers, allozyme electrophoresis and sequence analysis of the mtDNA.

(2) Glacial refugia along the margins of the ice sheets in the English Channel or in the southern North Sea have been proposed for anadromous salmonids (García-Marin *et al*, 1999; Verspoor *et al*, 1999). The euryhaline *P. microps* is capable of surviving in brackish water. Thus, if a glacial refugium existed in the area, it is not unlikely that an isolated population of *P. microps* managed to survive in the area as well. Analysis of (a) the geographic distribution of the various haplotypes (Templeton *et al*, 1992) and (b) past gene flow events (Beerli & Felsenstein, 2001) may shed more light on the existence and location of these putative refugia.

(3) During sea-level drops the connection between the Atlantic Ocean and the Mediterranean Sea was either severed or severely restricted, leading to the long-lasting isolation of populations, which evolved in allopatry. Moreover, considering the differences in migratory behaviour between Atlantic and Mediterranean *P. microps*, we hypothesise that recurrent genetic exchange between them must be limited. Thus, Mediterranean populations of the common goby have probably been evolving in allopatry for a long time and will most likely show a significant differentiation from their Atlantic conspecifics.

MATERIALS AND METHODS

Samples were taken by fishing with a hand net in tidal pools at low tide with the exception of the samples from the Schelde estuary (Doel), which were taken at the cooling water intake of the nuclear power plant. Sampling sites with number of fish screened for allozyme electrophoresis and mtDNA are listed in Table 3.1. Fish were frozen in dry ice or liquid nitrogen immediately after capture and stored in a -80°C freezer until analysis.

Table 3.1. Sampling sites of *Pomatoschistus. microps* along the European coasts including the numbers of fish screened with allozyme electrophoresis (AE) and SSCP analysis of mtDNA respectively.

Sampling site	Latitude	Longitude	Code	Period	AE	mtDNA
Trondheim (Norwegian Sea, Norway)	63° 24' N	10° 24 E	Tro	June 2000	50	26
Oban (Irish Sea, UK)	56° 24' N	5° 28' W	Ob	Aug. 1999	40	14
Galway (northeastern Atlantic Ocean, Ireland)	53° 16' N	9° 03'W	Ga	Aug. 1999	54	27
Texel (Wadden Sea, The Netherlands)	53° 00' N	4° 46' E	Tex	Nov. 2001	61	25
Dale (Celtic Sea, UK)	51° 42' N	5° 10' W	Da	May 1997	-	22
Doel (southern North Sea, Belgium)	51° 19' N	4° 16' E	Do	March 1998	57	16
Bray-Dunes (southern North Sea, France)	51° 04' N	2° 31' E	Bdu	Sept. 2001	-	26
Plymouth (English Channel, UK)	50° 22' N	4° 09' W	Ply	Nov. 1996	-	14
La Tremblade (Gulf of Biscay, France)	45° 46' N	1° 08' W	LT	Oct. 2000	53	32
Pérols (Gulf of Lions, France)	43° 34' N	3° 57' E	Per	Jan. 1999	53	20
Vaccarès Lagoon (Gulf of Lions, France)	43° 33' N	4° 31' E	Vac	Aug. 2000	-	20
Faro (northeastern Atlantic Ocean, Portugal)	37° 04' N	7° 55' W	Fa	Dec. 1998	56	29

Allozyme electrophoresis

A total of 364 individuals of *Pomatoschistus microps* from eight sampling sites was genotyped with cellulose acetate allozyme electrophoresis (Richardson *et al,* 1986) (CAGE) at thirteen putative allozyme loci (Table 3.2). Tris-maleate (pH = 7.8) and Tris -glycine (pH = 8.8) buffer systems were used as described by Hebert & Beaton (1989). Loci were stained according to recipes described by Hebert & Beaton (1989) and Richardson *et al* (1986). The slowest migrating locus was designated 1 or A according to the nomenclature of Shaklee *et al* (1990). The most common allele was called

'100' and other alleles were classified according to their mobility relative to allele 100 for the locus under study.

Table 3.2. Enzymes and their Enzyme Commission (E.C.) numbers and tissue employed. M = skeletal muscle; E = eye.

Enzyme	Locus (tissue)
Adenylate Kinase kinase (AK) [EC 2.7.4.3]	*AK** (M)
Aspartate aminotransferase (sAAT) [EC 2.6.1.1]	*AAT** (M)
Creatine Kinase kinase (CK) [EC 2.7.3.2]	*CK-A** (M)
Fumarate hydratase (FH) [EC 4.2.1.2]	*FH** (M)
Glucose-6-phosphate isomerase (GPI) [EC 5.3.1.9]	*GPI-A** (M,E), *GPI-B** (M, E)
L-Lactate dehydrogenase (LDH) [EC 1.1.1.27]	*LDH-A** (M), *LDH-B** (M), *LDH-C** (E)
Malate dehydrogenase (MDH) [EC 1.1.1.37]	*MDH-A** (M), *MDH-B** (M)
Phosphoglucomutase (PGM) [EC 5.4.2.2]	*PGM-1** (M), *PGM-2** (M)

Sequence and SSCP analysis of mtDNA

A total of 270 *Pomatoschistus microps* were screened from twelve sites for a 289 bp fragment from the mtDNA (Table 3.1). A fin clip was taken from each fish specimen and stored in 100% ethanol. DNA extraction was carried out with the DNEasy tissue kit (Qiagen International). Specific primers (Kocher *et al,* 1989) yielded a 300 bp fragment of the mitochondrial cytochrome b locus. PCR conditions were as follows: denaturation at 95°C for 3 min, annealing at 56°C for 45 s, elongation at 72°C for 45 s with a total of 35 cycli and a final elongation at 72° C for 7 min. The $MgCl_2$ concentration was 2 mM. Visible bands were cut out under UV, purified with the 'GFX PCR DNA and Gel Band Purification' kit (Amersham Biosciences, Little Chalfont, UK), eluted in 25 µl mQ distilled water and cloned into the PUC-57 vector. A second PCR was carried out on the PCR products and both strands were sequenced with standard M13 primers using the 'SequiTherm Excell II' kit (Epicentres Technologies, Madison, OH, USA). The reaction yielded a 289 bp fragment that was analysed on a LICOR 4200 automated sequencer (LI-COR GeneReadIR DNA system) with the AlignIR software (LI-COR). A search in BLAST confirmed that the right fragment had been amplified. Based on the sequences thus obtained from four different specimens of *P.*

microps specific internal primers were designed for screening populations using Single Stranded Conformational Polymorphism (SSCP) analysis (Orita *et al,* 1989). Primer sequences for the SSCP are: Pmic-cytbF 5'- ATT- TTG- GCT- CCT- TAC- TAG- GC- 3' and Pmic-cytbR 5'- TCA- GGG- AAG-AAC- GTA- GCC-3'. Conditions for the PCR were: denaturation at 97° C for 3 min, denaturation at 95° C for 45 s, annealing at 54° C for 45 s, elongation at 72° C for 45 s and final elongation at 72° C for 7 min with a total of 35 cycli at a concentration of 2 mM $MgCl_2$. Pre-made gels were employed (DNA analysis kit of Amersham Biosciences, cat. n° 17-1198-07). In the SSCP analysis each individual was run several times under identical conditions. A few individuals from each mobility group (depending on the SSCP mobility class frequency) were randomly chosen and their nucleotide sequence was determined on an automated DNA sequencer (LICOR 4200, Westburg). The SSCP was carried out at a temperature of 4° C with a run time of 2 h. at 600 V. Bands were visualised employing the DNA silver staining kit of Amersham Biosciences (cat. n°. 17-6000-30). The various haplotypes, which were visualised by the SSCP were subsequently sequenced. PCR conditions for the sequencing reactions were denaturation at 95° C for 3 min, denaturation at 95° C for 30 s, annealing at 54° C for 15 s, elongation at 70° C for 1 min with a total of 35 cycli, final elongation at 70° C for 7 min with a $MgCl_2$ concentration of 2 mM.

Data analysis

Genetic diversity was measured as the level of polymorphism, observed and expected heterozygosity and haplotype diversity. Allele frequencies and the observed and unbiased expected heterozygosity were calculated in GENETIX version 4.02 (Belkhir *et al*, 2001). Linkage disequilibrium and Hardy-Weinberg equilibrium were tested for significance in GENEPOP version 3.1 (Raymond & Rousset, 1995). Wright's single-locus F-statistics (Wright, 1951) were calculated from allele frequencies of all loci examined for each population according to Weir and Cockerham (1984) in GENETIX. Standard deviations of single-locus F_{ST} values were obtained by jacknifing over all populations according to Weir (1990) and the significance of the multilocus F_{ST} was assessed with permutation tests (1000 replicates).

Sequences were aligned in CLUSTALW version 1.7 (Thompson *et al,* 1994). Genetic diversity, measured as haplotype diversity (h) and nucleotide diversity (π) (Nei, 1987) was computed in DNASP version 3.51 (Rozas & Rozas, 1999). A linear regression between log (x+1) transformed data on the heterozygosity, resp. haplotype diversity against latitude was computed in

STATISTICA version 6.0 (Statsoft 2001). Because visual inspection of the sequences revealed a strongly unequal distribution of mutation sites throughout the cytochrome b fragment, the program PUZZLE (Strimmer & von Haeseler 1999) was used for calculating the parameter α of the gamma distribution. Pair-wise genetic distances were computed in ARLEQUIN version 2.0 (Schneider *et al,* 2000) according to the model of Tamura-Nei (1993), which takes into account unequal nucleotide frequencies and unequal mutation rates within the fragment. Significance was assessed with permutation tests (1000 replicates). A sequential Bonferroni test was applied to correct significance levels for multiple testing (Rice, 1989). The distribution of pair-wise differences between haplotypes (mismatch distribution) (Rogers & Harpending, 1992) with its parameters τ, θ_0 and θ_1 and Tajima´s D-statistic (Tajima, 1989a) were also computed in the program ARLEQUIN and tested for significance with permutation tests (1000 replicates). For assessing whether any indications for group structure could be observed, we performed a Multidimenisonal Scaling Analysis (MDS) on the pair-wise Tamura-Nei genetic distances in the program STATISTICA. Subsequently, samples were grouped and subjected to a hierarchical analysis of variance (AMOVA) (Excoffier *et al*, 1992) in the program ARLEQUIN in order to ascertain whether the group structure as observed in the MDS was significant. A minimum spanning network was calculated in ARLEQUIN and used as a nested design for constructing a Nested Clade Analysis (NCA) network. Nested Clade Analysis (Templeton *et al,* 1995) tests for geographical associations between haplotypes and groups of haplotypes on different hierarchical levels and allows distinguishing between historical events such as range expansion and past fragmentation and contemporary gene flow to explain the observed genetic structure. Significance of clade distances and nested clade distances was calculated with permutation tests as implemented in the program GEODIS (Posada *et al,* 1999) and interpreted with the inference key according to Templeton (1998). However, as has been pointed out by Knowles & Maddison (2002), Nested Clade Analysis does not provide a statistic test of alternative interpretations. Thus, as a complementary approach to NCA, we employed a coalescence-based method for assessing directions of past gene flow between populations with the program MIGRATE version 1.5 (Beerli, 2002). This program calculates maximum likelihood estimates for migration rates and effective population sizes based on coalescence theory, allowing for asymmetric migration rates and different subpopulation sizes (Beerli & Felsenstein, 2001). Parameter values were ten short chains with 500 steps and 10.000 sampled genealogies and three long chains with 5000 steps and 100.000 sampled genealogies.

RESULTS

Genetic diversity analysis

Allele frequencies at the various loci, observed and expected heterozygosity, mean number of alleles per locus and the proportion of polymorphic loci are listed in Appendix 3.1. Loci *AAT**, *GPI-A**, *GPI-B**, *PGM-1** and *PGM-2** are polymorphic in all samples with the exception of Pérols and Trondheim, which are completely fixed for the same allele at locus *GPI-A**. Loci *LDH-A** and *LDH-B** are monomorphic in all samples except for Doel, where two alleles were recorded. *MDH-A** is fixed for the same allele in most samples, with the exception of Texel, Pérols and La Tremblade, where a rare second allele was recorded. *MDH-B** is fixed for the same allele in all samples with the exception of Galway, where three individuals were found which were heterozygous for two other alleles than the common one (*MDH-B*75/115* as opposed to *MDH-B*100/100*). No differences at other loci were recorded in these fish. Loci *AK**, *CK**, *LDH-C** and *FH** are monomorphic for the same allele in all samples. The lowest and highest level of heterozygosity and average number of alleles per locus are found in the samples at the northern- and southernmost sampling sites (respectively Trondheim and Faro). Observed heterozygosity in Trondheim amounts to 0.066 and the mean number of alleles per locus is 1.23. In the sample from Faro on the contrary, observed heterozygosity is an order of magnitude higher, amounting to 0.135, while the mean number of alleles is 1.77. The samples from Doel, Texel, Galway and Oban show similar values for the average number of alleles per locus. No significant departures from Hardy-Weinberg proportions were observed with the exception of locus *PGM-2** in the sample from La Tremblade ($P < 0.001$) and locus *MDH-B** ($P < 0.001$) in the Galway. No significant linkage disequilibrium was observed with the exception of loci *GPI-A** and **AAT* in the sample from Faro ($P = 0.006$).

Table 3.3. Observed (H_o) and unbiased (H_e) expected heterozygosity (Nei, 1978) with standard deviation, mean number of alleles per locus (MNA), number of mtDNA haplotypes (N_h), number of unique haplotypes (N_h unique), haplotype (h) and nuceotide (π) diversity with standard deviation and % of occurrence of the most common haplotype (% MCH).

Site	H_e	H_o	MNA	h	N_h	N_h unique	π	% MCH
Tro	0.075 (0.152)	0.066 (0.138)	1.231	0	1	0	0	H14 -100%
Ob	0.106 (0.185)	0.097 (0.169)	1.385	0.143 (0.119)	2	1	0.0010 (0.0008)	H4 - 93%
Ga	0.108 (0.161)	0.117 (0.183)	1.692	0.484 (0.061)	3	2	0.0017 (0.0004)	H4 - 70%
Tex	0.121 (0.200)	0.114 (0.186)	1.615	0.357 (0.115)	4	2	0.0022 (0.0011)	H4 - 80%
Da	-	-	-	0.177 (0.106)	3	2	0.0012 (0.0007)	H1- 91%
Do	0.124 (0.201)	0.125 (0.207)	1.539	0.608 (0.130)	5	1	0.0060 (0.0015)	H4 - 63%
Bdu	-	-	-	0.557 (0.109)	5	2	0.0059 (0.0016)	H4 – 65 %
Ply	-	-	-	0.791 (0.089)	6	3	0.0081 (0.0015)	H1- 40%
LT	0.120 (0.195)	0.098 (0.156)	0.308	0.633 (0.090)	7	3	0.0048 (0.0008)	H1- 58%
Per	0.081 (0.157)	0.076 (0.155)	1.539	0.653 (0.076)	5	2	0.0032 (0.0007)	H14 -50%
Vac	-	-	-	0.606 (0.062)	4	0	0.0028 (0.0007)	H14 -50%
Fa	0.139 (0.203)	0.135 (0.193)	1.769	0.857 (0.062)	16	13	0.0081 (0.0013)	H6 - 38%

Thirty-eight mtDNA haplotypes were detected among all samples (Appendix 3.2). Sequences of the various haplotypes are deposited at the EMBL sequence database; accession numbers of the various haplotypes and mutation sites are presented in Appendix 3.2. All differences between haplotypes were due to substitutions (transition: transversion ratio = 6.03) and no indels were recorded. The distribution of the various haplotypes per sample is presented in Appendix 3.3. A similar geographic difference in haplotype diversity is observed, with the Faro sample showing the highest level of haplotype diversity and number of haplotypes ($h = 0.857$, $N_h = 16$), while in the sample from Trondheim a single haplotype was recorded ($h = 0$, $N_h = 1$). The sample from Faro is not only the most diverse, it has also a very high number of unique haplotypes (13 out of 16 among 29 fish) (Table 3.3).

Genetic diversity and latitude

Allozyme data show a negative correlation between latitude and level of heterozygosity, although not significant ($R^2 = 0.231$, $P = 0.26$). An outlier in the regression is the sample from Pérols, revealing a much lower degree of heterozygosity than expected for its latitude. When only the

Atlantic samples are taken into account, the regression between heterozygosity and latitude approaches significance ($R^2 = 0.56$, $P = 0.052$) (Fig. 3.2a). No latitudinal trend in allele frequencies was observed at any polymorphic locus ($P > 0.05$).

The mtDNA data reveal a similar negative correlation between haplotype diversity and latitude, but in this case the regression is significant ($R^2 = 0.69$, $P = 0.0008$) with a haplotype diversity ranging from 0 (only 1 haplotype in the sample from Trondheim) to 0.857 (16 haplotypes in the Portuguese sample) (Fig. 3.2b).

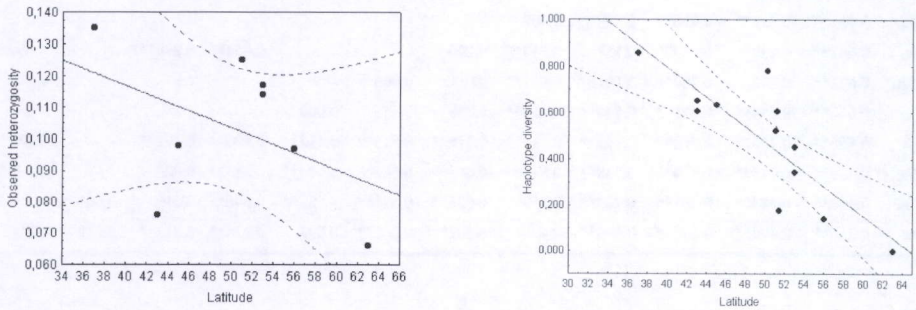


Figure 3.1. *Pomatoschistus microps*: regression of heterozygosity (a) and haplotype diversity (b) against latitude for all samples.

Genetic structure

Allozyme data show that the multi-locus F_{ST} value across all populations amounts to 0.247 (Appendix 3.1). Permutation tests on single-locus F_{ST} values showed that samples are significantly differentiated at four out of five polymorphic loci: *PGM-2*, GPI-A*, GPI-B** and *AAT** (Appendix 3.1). The highest degree of differentiation is found at locus *AAT**. Calculation of pair-wise F_{ST} values revealed significant population differentiation between most sampling sites except between Texel and Doel (Table 3.4). Within the northeastern Atlantic Ocean the largest values of pair-wise distances are found between the sample from Oban and all the others. This difference is of the same order as between the Mediterranean sample (Pérols) and the Atlantic samples (Table 3.4).

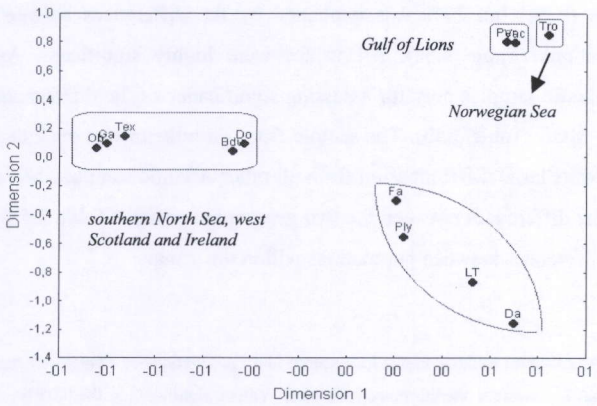
Table 3.4. *Pomatoschistus microps*: pair-wise F_{ST} values (Weir & Cockerham, 1984) based on allozymes (above diagonal) and genetic distance (Tamura-Nei, 1993) (below diagonal) for the mtDNA haplotypes among all samples. Values significant at the 0.05 and 0.01 level after sequential Bonferroni correction are indicated with * and ** respectively. For sample abbreviations see Table 3.1.

	Tro	Ob	Ga	Tex	Da	Do	Bdu	Ply	LT	Per	Vac	Fa
Tro	0.000	0.312**	0.097**	0.325**	-	0.268**	-	-	0.308**	0.333**	-	0.155**
Ob	0.955**	0.000	0.291**	0.346**	-	0.310**	-	-	0.396**	0.308**	-	0.263**
Ga	0.892**	0.151	0.000	0.270**	-	0.202**	-	-	0.178**	0.420**	-	0.039**
Tex	0.862**	0.009	0.137*	0.000	-	0.011	-	-	0.067**	0.282**	-	0.140**
Da	0.961**	0.935**	0.910**	0.888**	0.000	-	-	-	-	-	-	-
Do	0.642**	0.158	0.239**	0.128	0.717**	0.000	-	-	0.054**	0.298***	-	0.092**
Bdu	0.622**	0.122	0.200**	0.101	0.671**	-0.032	0.000	-	-	-	-	-
Ply	0.690**	0.553**	0.610**	0.544**	0.285**	0.229	0.235	0.000	-	-	-	-
LT	0.755**	0.748**	0.768**	0.733**	0.113	0.518**	0.515**	0.112	0.000	0.402**	-	0.051**
Per	0.336**	0.771**	0.774**	0.738**	0.848**	0.518**	0.546**	0.574**	0.684**	0.000	-	0.343**
Vac	0.360**	0.787**	0.785**	0.749**	0.859**	0.529**	0.553**	0.583**	0.690**	-0.050	0.000	-
Fa	0.566**	0.496**	0.547**	0.496**	0.428**	0.242**	0.262**	0.093	0.224**	0.513**	0.518**	0.000

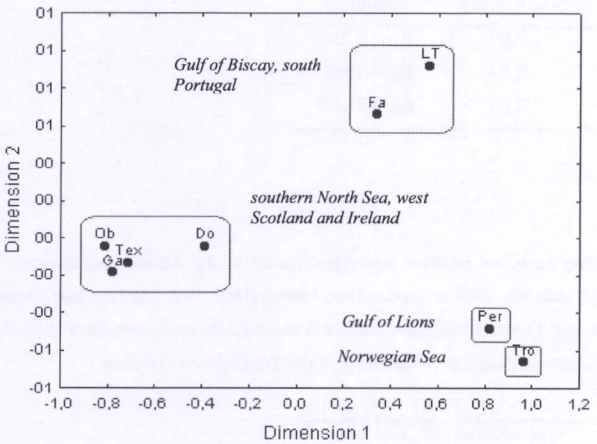
Analysis of the mtDNA data for population differentiation showed that most pair-wise genetic distances are highly significant (Table 3.4). No differentiation between the adjacent sampling sites of Pérols and Vaccarès was recorded. The samples from the North Sea and the English Channel (resp. Doel, Bray-Dunes, Texel, Oban, Galway) are dominated by the same haplotype H4 and are not significantly differentiated. The samples from the Gulf of Biscay and the southwestern British coast (resp La Tremblade, Plymouth and Dale) are closely related as well, but differ from the North Sea samples, H1 being the most common haplotype among these samples.

Although less samples were analysed for allozymes than for mtDNA, the multidimensional Scaling Analysis clearly revealed a similar group structure for both markers (Fig. 3.2a and b). The samples from the southern North Sea (Texel, Doel, Bray-Dunes) grouped together with those from western Scotland and Ireland (Oban and Galway). A second group comprised the English Channel, the Celtic Sea, the Gulf of Biscay and southern Portugal (Dale, Plymouth, La Tremblade, Faro). The third group consisted of the western Mediterranean Sea (Gulf of Lions: Pérols and Vaccarès), including also the sample from Trondheim (Fig. 3.2a). This is due to the occurrence of the same haplotype (H14) in Mediterranean and Norwegian common gobies at high frequencies (ranging from 50% to 100%) (Appendix 3.3). The grouping of the western Mediterranean Sea and

Trondheim for the allozymes (Fig. 3.2b) results from similarities in frequencies at loci *GPI-A** and *GPI-B** between these samples; both samples are fixed for *GPI-A*100* and have higher frequencies of at allele **90* at locus *GPI-B** than the other samples (Appendix 3.3).



(a)



(b)

Figure 3.2. *Pomatoschistus microps*: Multidimensional Scaling Analysis based on (a) the matrix of pair-wise Tamura-Nei genetic distances of cytochrome b samples (Stress value = 0.0002); (b) pair-wise F_{ST} values of allozyme screened samples. Stress value = 0.0000.

Because of the larger amount of sampling sites available, the mtDNA samples were grouped according to the structure observed in the Multidimensional Scaling Analysis for performing a hierarchical analysis of variance (AMOVA). An AMOVA was performed between two groups of samples: Atlantic versus Mediterranean. The largest amount of variation was due to differences between populations within groups (40%) but 27% was explained by the differences between Atlantic and Mediterranean populations (Table 3.5a). All values were highly significant. An AMOVA was also performed on Atlantic samples only for assessing significance of the differences between the groups observed in the MDS (Table 3.5b). The sample from Trondheim was excluded from this hierarchical analysis due to its large differentiation from all other Atlantic samples. More than half of the variation is due to the differences between the two geographic groups (Table 3.5b), while only 8.38 % is explained by differences between populations within the groups.

Table 3.5a: Hierarchical analysis (AMOVA) based on mtDNA haplotypes scored in all populations of *Pomatoschistus microps*. Group 1 = Atlantic samples. Group 2 = western Mediterranean samples. Values significant at the 0.05 level are indicated with an *.

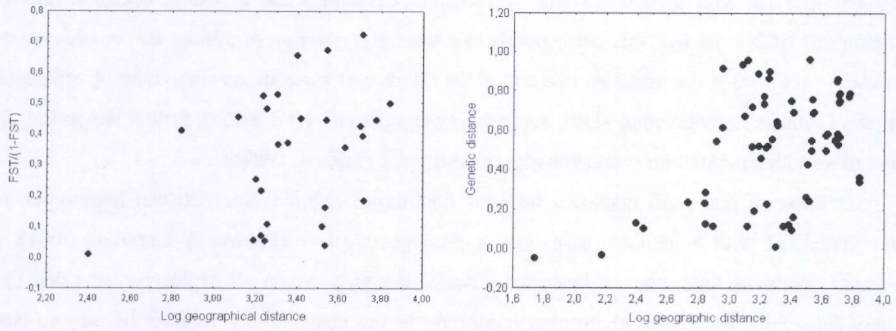
Source of variation	% of variation	F-statistics
Between groups	27.39	$F_{CT} = 0.274$*
Among populations within groups	40.09	$F_{SC} = 0.552$*
Within populations	32.52	$F_{ST} = 0.675$*

Table 3.5b: Hierarchical analysis (AMOVA) based on mtDNA haplotypes scored in the Atlantic populations of *Pomatoschistus microps*. Group 1: southern North Sea, western Scotland and Ireland (Doel, Bray-Dunes, Texel, Oban, Galway). Group 2 = Celtic Sea, English Channel, Gulf of Biscay and southern Portugal (Dale, La Tremblade, Plymouth, Faro). Values significant at the 0.05 level are indicated with an *. The sample from Trondheim is excluded.

Source of variation	% of variation	F-statistics
Between groups	54.27	$F_{CT} = 0.543$*
Among populations within groups	8.38	$F_{SC} = 0.183$*
Within populations	37.35	$F_{ST} = 0.626$*

Isolation-by-distance

A plot of (a) allozyme-based pair-wise F_{ST} and (b) Tamura-Nei genetic distances (mtDNA) versus the geographic distance shows a clear pattern of isolation-by-distance (Fig. 3.2a and b). However, a Mantel test performed on pair-wise F_{ST}/ (1-F_{ST}) for all allozyme samples was not significant (r = 0.32, P = 0.081). On the other hand, a Mantel test based on the pair-wise mtDNA distances was significant (r = 0.33, P = 0.037).



(a) (b)

Fig. 3.2: *Pomatoschistus microps*: allozyme-based pair-wise F_{ST} values (a) and pair-wise genetic distances based on mtDNA sequences (b) versus log-transformed coastal distance for all samples.

Geographic distribution of the haplotypes

Pooling of all samples revealed three common haplotypes (H1,19.5 %; H4, 29.9 % and H14, 18.0 % frequency occurrence in the total sample size), and accompanied by a fairly high number of rare to very rare variants (Appendix 3.3). Only one of the haplotypes (H14) is shared between the Mediterranean and Atlantic samples. Surprisingly, this is also the only haplotype occurring in the Trondheim sample. Only two other individuals carrying H14 were found in the Atlantic basin (Doel). Haplotype H4 seems to be restricted to the samples from the English Channel and the southern North Sea (Plymouth, Bray-Dunes, Doel and Texel) and the northwestern British coasts

(Galway and Oban); it was not recorded in Faro or La Tremblade. Haplotype H1 is the most common haplotype in La Tremblade and Dale. It occurs also in Texel, Doel and Plymouth, albeit at a low frequency.

Phylogeographical analysis

Tests of the goodness of fit revealed that the model of a sudden population expansion for the pair-wise distribution of the *Pomatoschistus microps* haplotypes could not be rejected. The value for τ amounted to 3.10, with $\theta_0 = 0.133$ and $\theta_1 = 566.95$. Assuming the generally accepted mtDNA mutation rate of 2% per Ma, this corresponds to a time of expansion of 536 ka BP, employing the formula $\tau = \mu T$ with μ the mutation rate and T the number of generations since time of expansion. Tajima's D statistic amounted to -1.40, approaching significance ($P = 0.054$), further supporting the model of a sudden population expansion after a bottleneck (Tajima, 1989b).

Because of the small distances between haplotypes relationships between haplotypes are better visualised with a network than with a phylogenetic tree (Posada & Crandall, 2001). A Minimum Spanning Tree, used as basis for a nested design between all haplotypes revealed four clusters (Fig. 3.3), each with a common haplotype in the center and a number of rare variants radiating. The Mediterranean cluster, containing only four haplotypes, is connected with the Atlantic cluster through haplotype H14, the only haplotype which is shared between Mediterranean and Atlantic samples.

Nested Clade Analysis

The null hypothesis of no geographic association of the haplotypes was rejected at all clade levels for several clades. The overall results of the Nested Clade Analysis suggest restricted gene flow with isolation-by-distance. On a smaller time scale, the 1-step clades containing the Atlantic haplotypes H1 and H4 suggested a recent long distance dispersal or a contiguous range expansion. In order to discriminate between these hypotheses, sampling design should be refined (Table 3.6).

Table 3.6: Phylogeographical inferences from the Nested Clade Analysis (Templeton, 1998) of *P. microps*. Only clades where the null hypothesis could be rejected are represented. For the nested design, see Fig. 3.3.

Clade	common haplotype / geographical distribution	Inference
1-3	H1 - south and west of English Channel	Contiguous range expansion or long distance colonisation (Inadequate sampling design)
1-5	H4 - North Sea and north western British coasts	Contiguous range expansion or long distance colonisation (Inadequate sampling design)
2-1	H1 - south and west of English Channel	Contiguous range expansion
2-3	H14 - Mediterranean Sea and Trondheim	Restricted gene flow with isolation-by-distance
2-4	H6 - Atlantic coasts of south Europe, Gulf of Biscay, southwest Britain	Restricted gene flow with isolation-by-distance
3-1	Total cladogram	Restricted gene flow with isolation-by-distance

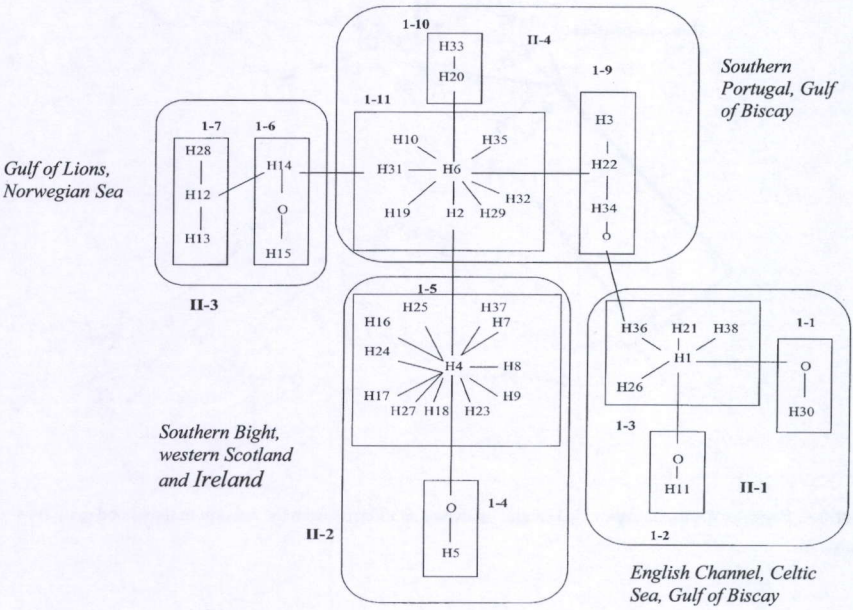


Figure 3.3. Nested design for *P. microps* haplotypes with the geographic location of the various clusters.

Gene flow analysis

Maximum likelihood gene flow analysis on the Atlantic samples (Trondheim excluded) showed (1) a northward gene flow from the Faro population as far north as the Schelde estuary, (2) a northward gene flow through the Southern Bight and (3) a northward gene flow from the Southern Bight towards Scotland and Ireland (Fig. 3.4).

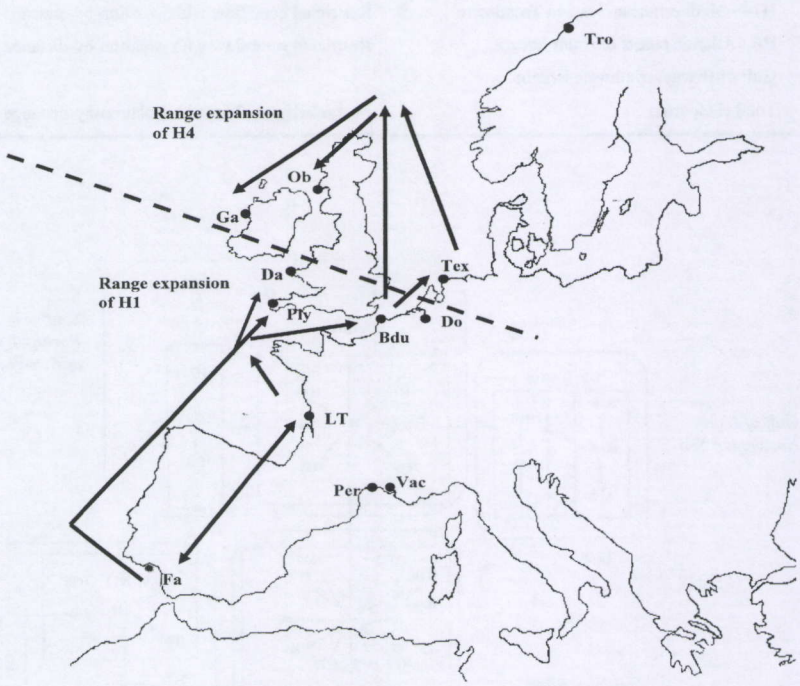


Figure 3.4. *Pomatoschistus microps* : Past migration events as inferred from the maximum likelihood gene flow analysis.

Most striking is the remarkable congruence in global results on genetic structure for mitochondrial and allozyme markers: (1) a significant degree of population differentiation in *Pomatoschistus microps* throughout its distributional range, (2) a differentiation between northern and southern *P.*

minutus populations in the northeastern Atlantic basin, (3) a strong correlation between the degree of genetic variation and latitude, and (4) a pattern of isolation-by-distance. The mtDNA data yield a finer resolution, showing (1) the different histories for Atlantic and Mediterranean *P. microps* in the Minimum Spanning Tree and (2) the striking genetic discontinuities around the British Isles. However, the phylogeographic patterns of *P. microps* seem to be very complex.

Loss of variation during postglacial range expansion or natural selection?

The marine common goby shows reduced diversity with latitude, a pattern typical for populations inhabiting formerly glaciated areas in terrestrial and freshwater species (Merilä *et al,* 1996; Bernatchez & Wilson, 1998; Hewitt, 2000). Such a correlation is usually attributed to historical factors due to the loss of alleles (founder events) during range expansion following deglaciation (Hewitt, 2000). On the other hand, selection might play a role as well in maintaining latitudinal differences in the levels of genetic diversity (Mishmar *et al,* 2003). A similar effect is found at two independent markers in *P. microps*. Given the unambiguous congruence of two independent markers in the common goby, we favour the hypothesis that neutral variation provides the main explanation and we suggest that genetic variation was lost during the postglacial expansion of *P. microps* into formerly glaciated areas.

Phylogeographic patterns on the European Continental Shelf and in the Mediterranean Sea

The shallow phylogeographic structure of *Pomatoschistus microps* is best described by category V of Avise (2000), which refers to a shallow gene tree with varied lineage distributions: widespread common lineages ('Atlantic') are combined with 'private' closely related lineages ('Mediterranean-Trondheim'). The 'Atlantic' clusters each show a star like phylogeny with a central, common and widespread (ancestral) haplotype and a large number of rare (local) variants, suggesting a population expansion after contraction.

The western Mediterranean Sea

It is assumed that the origin of the Atlanto-Mediterranean sand gobies, to which the common goby belongs, lies within the Mediterranean Sea, where the oldest fossil remains have been found (Simonovic, 1999). Huyse (2002) proposed a speciation event within the sand goby group triggered by reflooding of the Mediterranean after the Messinian salinity crisis. Currently, the common goby has a very restricted distribution in the Mediterranean Sea, occurring only in the Gulf of Lions, where our samples were collected, and in Corsica (Bouchereau & Guelorget, 1997).

A possible scenario for explaining the phylogeographic patterns might be that a Mediterranean population H14 *P. microps* dispersed into the Atlantic and gave rise to all the other Atlantic haplotypes, while those that remained behind diverged into the other Mediterranean haplotypes when colonising lagoons and estuaries. The fact that only the western part of the Mediterranean Sea is inhabited by *P. microps* could be due to ecological constraints. *P. microps,* being an estuarine-marine species, may not tolerate the higher temperature and salinity which are typically found in the central and eastern Mediterranean basin.

An alternative hypothesis is that the various haplotypes of *P. microps* originated in the Atlantic Ocean along the Iberian coast. Haplotype and nucleotide diversity in the sample from Faro are highest, pointing to an older age of this population. Moreover, the MST suggests H6, the most common haplotype in Faro, as ancestral. According to this interpretation, an Atlantic population of H14-*P. microps* colonised the western Mediterranean Sea, giving rise to the endemic haplotypes H12, H13, H15 and H28. Considering the low amount of diversity and the low level of divergence between the haplotypes, colonisation of the lagoons and estuaries in the Gulf of Lions is probably a relatively recent event from a small and/or genetically homogeneous ancestral population.

The northeastern Atlantic Ocean: a postglacial range expansion in two phases

Haplotype H14 occurs in high frequencies at the edges of *P. microps*' distributional range, while it is almost absent in the other areas sampled. The grouping of the samples from Trondheim and the Mediterranean Sea at the 2-step level in the NCA (Fig. 3.3) suggests that the range expansion of H14 is an older event, which preceded dispersal of the haplotypes H1 and H4. A possible scenario could be the displacement of H14 by a more recent range expansion of H1 and H4. Such a scenario

was proposed for explaining phylogeographic patterns in *Hydrobia* sp. (Wilke & Pfenniger, 2002), where two subspecies are geographically separated by another species, due to a range shift or expansion of the latter. Alternatively, individuals with haplotype H14 may correspond to long-distance dispersers as described by Ibrahim *et al* (1996). Long-distance migration of rare variants leads to the formation of pocket populations in advance of the arrival of the main population, which may persist for hundreds of generations. Long-distance dispersers may have expanded rapidly and filled up available niches before others, leading to edge populations with reduced variability (Hewitt, 2000). The edge populations of *P. microps* in the Norwegian Sea and the Gulf of Lions have indeed a lower variability compared to the others.

Evidence for recolonsiation events from multiple refugia

That the highest level of diversity was found in the southernmost population from Faro with the two markers, may reflect an older age of this population. This is concordant with the idea of a northward range expansion of *P. microps* from a refugium along the Atlantic coast of the Iberian peninsula as proposed for salmon (Consuegra *et al*, 2002) and brown trout (García-Marín *et al*, 1999). Because the common goby needs shallow areas for breeding, the ice-covered of northwestern Europe were unsuitable habitats. During glaciation the winter sea ice came down as far south as the coasts of northern Spain while the Portuguese coast remained ice-free (Andersen & Borns, 1997). While the present distribution of *P. microps* must be the result of its northward range expansion after the last ice age, which ended about 10 ka BP, the mismatch analysis indicates that the origin of the Atlantic haplotypes has to be of a considerably more ancient origin. With an estimate of 536 ka BP, this could correspond with the onset of an earlier warm interglacial period during the Cromerian complex (Prokopenko *et al,* 2002). *P. microps* must have experienced similar range contractions and expansions during earlier glaciations and interglacials.

Yet, as revealed by the nested clade and the gene flow analysis, the finer phylogeographic patterns within the northeastern Atlantic Ocean seem to be rather complex, with the striking differences in haplotype distribution between northwestern and southwestern British populations.

A glacial refugium in the southern North Sea where a H4 - population survived?

The gene flow analysis indicates the Gulf of Biscay as the origin of the *P. microps* that have migrated to southwestern Britain (H1), while the Southern Bight is suggested to be the origin of the *P. microps* which colonised Scotland and Ireland (H4) (Fig. 3.4). If this is correct, then a population of H4 - *P. microps* must have survived in a glacial refugium in the area. Glacial refugia in the area have been proposed for salmonids. For example, García-Marín *et al* (1999) proposed the drainage systems of the major rivers, which flowed through the English Channel during lowered sea-level (Gibbard, 1988) as a glacial refugium for brown trout. Verspoor *et al* (1999) suggested a glacial lake in the southern North Sea as a refugium for Atlantic salmon. The distribution of the *P. microps* haplotypes points to a refugium in the Southern Bight of the North Sea, rather than in the English Channel. *P. microps* is euryhaline and capable of surviving in brackish water areas, and occurs commonly upstream in rivers (Claridge *et al*, 1985; Maes *et al* 1998a). Thus, physiologically the species must have been able to survive in a glacial lake. Hence, the English Channel and the southern North Sea may consist of a zone of secondary contact between H1 and H4-dominated *P. microps*. The fact that a unique haplotype, H17, was found in the Irish sample in an appreciable frequency (29 %) suggests that this population may also have been isolated from all the others for some time. Hynes *et al* (1996) recorded distinct mtDNA haplotypes in Irish brown trout and suggested a complex recolonisation process from multiple refugia in this area.

Alternatively, both H1 and H4 may have survived the Weichselian glaciation in the southern refugium along the Portuguese coast. The break between the haplotypes H1 and H4 coincides with the biogeographical boundary between the warmer Lusitanian province (southern British and Irish coasts and south of the English Channel) and the colder Boreal province (North Sea and the coasts of west Scotland and Ireland) following Ekman (1953). Thus, the recurrent distribution of the haplotypes around the British Isles could also be explained in terms of temperature-dependent selection. According to this interpretation, the cold-adapted H4-*P. microps* may have been the first to undertake a northward range expansion upon the re-establishment of the North Atlantic Drift, around 10 ka BP. A second wave of common gobies, carrying H1 might have arrived at the southwestern English coast at a later stage. If indeed H1 is performs better in warmer water, they may have displaced the H4 *P. microps* in the English Channel and the Celtic Sea. Comparing with the survey of Jones & Miller (1966) we notice that the areas where *P. microps* carries out a thermal migration are dominated by H4, while H1 is most common in slightly warmer water. Al-Hassan *et*

al (1987) screened allozyme frequencies at loci *GPI-A** and *GPI-B** for *P. microps* from 20 sites across the British Isles and found a significant differentiation in allele frequencies between 1) Atlantic coasts and 2) English Channel and southern North Sea at both loci. This was correlated with the mean sea temperatures, suggesting the effect of temperature-dependent selection.

However, invoking natural selection for explaining the haplotype distribution in the common goby leaves us with the question as to why haplotype H14 is common both in Trondheim and in the Mediterranean Sea, two areas with a marked difference in temperature regime. A similar grouping structure is indicated by the two markers, as revealed by the MDS, clearly dividing the Atlantic samples in an northern and southern group. This suggests similar underlying mechanisms, pointing to historical factors rather than recurrent environmental effects.

Genetic differentiation and implications for recurrent levels of gene flow

Any large-scale contemporary dispersal in the common goby is likely to depend mainly on its pelagic larval stage. Consequently, this must be subject to the influence of the main current patterns in its distributional area.

The differentiation between the *P. microps* from the Portuguese coast (Faro) and the western Mediterranean Sea is striking, considering that these geographically close populations did not share one single haplotype among them on a total of 69 fish screened. Allele frequencies differ also markedly between these two samples and they share few rare alleles. This suggests that current gene flow between Atlantic and Mediterranean *P. microps* must be limited or even absent. Numerous examples of significant differentiation between Atlantic and Mediterranean populations have been described, and been attributed to the presence of the Almería-Oran Oceanic Front (AOOF) in the Alboran Sea, the most western part of the Mediterranean Sea (Quesada *et al*, 1995; Naciri *et al*, 1999; Zane *et al*, 2000). The AOOF represents the boundary between inflowing Atlantic water and the warmer, saline Mediterranean water (Tintore *et al*, 1988). Alternatively, lack of contemporary gene flow between Atlantic and Mediterranean *P. microps* may also be explained by the fact that Mediterranean *P. microps* complete their entire lifecycle in the estuarine environment, contrary to Atlantic *P. microps,* which carry out spawning and thermal migrations (reviewed in Bouchereau & Guelorget, 1997). Thus, even if Atlantic *P. microps* manage to cross the AOOF, the probability of genetic exchange with resident Mediterranean populations must be small.

Along the northeastern Atlantic coasts a pattern emerges of restricted gene flow and isolation-by-distance as shown with allozymes and mtDNA. Gene flow between the sand gobies from Galway and Oban may be maintained by the North Atlantic Drift, which flows northwest along the Irish and Scottish coasts. Gene flow between the North Sea sand gobies and those from western Scotland and Ireland, may be enhanced by the branching of the North Atlantic Drift into the North Sea (Turrell, 1992). The lack of differentiation between the Gulf of Biscay and the Celtic Sea is congruent with the results for European hake from Lundy *et al* (1999) and Dover sole (Exadactylos *et al,* 1998). The Shelf Edge Current, flowing from northwestern Africa to Norway along the edge of the Continental Shelf, provides a means for dispersal of planktonic fish larvae between the Bay of Biscay and the western coasts of the British Isles (Bartsch & Coombs, 1997). A northward residual current flows through the English Channel into the Southern Bight of the North Sea, which might explain the lack of differentiation between the samples from the English Channel and the Southern Bight.

CONCLUSIONS

Pomatoschistus microps is significantly differentiated throughout its distributional range with the highest degree of differentiation found between Atlantic and Mediterranean populations. This points to either a barrier for dispersal across the Almería-Oran front. Alternatively, the differences in migratory behaviour between Atlantic and Mediterranean *P. microps* might prevent genetic exchange. The lack of genetic differentiation between samples from the North Sea, the English Channel and the northwestern coasts of Scotland and Ireland is consistent with a larval dispersal via oceanic currents along the coasts of continental Europe and southern England.

The congruence of allozymes and mitochondrial DNA points to the importance of historical factors for explaining recurrent patterns of population structure. The negative correlation between genetic diversity and latitude suggests a loss of diversity during postglacial range expansion due to a founder event, while the star-like phylogeny of the Atlantic haplotype clusters suggest a population expansion after a contraction. Phylogeographic patterns in this species are complex but suggest that the source population, from where the species expanded its range northward, inhabited the Atlantic coast of the Iberian peninsula. The North Sea and the coasts of western Scotland and

Ireland have probably been recolonised by a population surviving in a glacial refugium in the southern North Sea.

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APPENDICES

Appendix 3.1. Number of individuals (N), allele frequencies, observed and non-biased expected heterozygosity (H_o and H_e) with standard deviation. MNA: mean number of alleles; P: proportion of polymorphic loci (0.99 level). F_{ST}: single-locus F_{ST} values across all samples. Significant F_{ST} values are indicated with an *.

Allele	Locus	Samples								F_{ST}
		Tro	Ob	Ga	Tex	Do	LT	Per	Fa	
*AAT**	*90	0.000	0.000	0.000	0.000	0.000	0.012	0.010	0.020	0.374*
	*100	0.670	0.136	0.878	0.655	0.673	0.952	0.186	0.878	
	*150	0.330	0.864	0.132	0.345	0.327	0.036	0.804	0.122	
N		47	33	53	58	26	42	51	49	
H_e		0.447	0.239	0.231	0.456	0.449	0.093	0.322	0.253	
H_o		0.404	0.212	0.264	0.414	0.500	0.095	0.275	0.286	
*GPI-A**	*90	0.000	0.419	0.213	0.066	0.047	0.189	0.000	0.269	0.142*
	*100	1.000	0.581	0.787	0.934	0.953	0.802	1.000	0.694	
	*113	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.037	
N		49	37	54	61	53	53	52	54	
H_e		0.000	0.494	0.338	0.124	0.091	0.324	0.000	0.448	
H_o		0.000	0.351	0.426	0.131	0.094	0.321	0.000	0.426	
*GPI-B**	*65	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.009	0.148*
	*90	0.776	0.514	0.546	0.418	0.361	0.406	0.942	0.510	
	*100	0.224	0.486	0.454	0.574	0.639	0.566	0.058	0.462	
	*116	0.000	0.000	0.000	0.000	0.000	0.028	0.000	0.019	
N		49	36	54	61	54	53	52	52	
H_e		0.352	0.507	0.500	0.500	0.466	0.519	0.110	0.532	
H_o		0.327	0.528	0.537	0.443	0.574	0.491	0.077	0.500	
*LDH-A**	*65	0.000	0.000	0.000	0.000	0.021	0.000	0.000	0.000	0.012
	*100	1.000	1.000	1.000	1.000	0.979	1.000	1.000	1.000	
N		50	37	53	61	47	52	51	53	
H_e		0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	
H_o		0.000	0.000	0.000	0.000	0.043	0.000	0.000	0.000	
*LDH-B**	*100	1.000	1.000	1.000	1.000	0.989	1.000	1.000	1.000	0.001
	*105	0.000	0.000	0.000	0.000	0.011	0.000	0.000	0.000	
N		50	37	53	61	46	52	51	53	
H_e		0.000	0.000	0.000	0.000	0.022	0.000	0.000	0.000	
H_o		0.000	0.000	0.000	0.000	0.022	0.000	0.000	0.000	
*MDH-A**	*80	0.000	0.000	0.000	0.008	0.000	0.000	0.009	0.000	-0.003
	*100	1.000	1.000	1.000	0.992	1.000	0.990	0.991	1.000	
	*150	0.000	0.000	0.000	0.010	0.000	0.010	0.000	0.000	
N		49	40	52	61	57	52	53	56	
H_e		0.000	0.000	0.000	0.016	0.000	0.019	0.019	0.000	
H_o		0.000	0.000	0.000	0.016	0.000	0.019	0.019	0.000	

Appendix 3.1 (continued)

Locus	Allele	Samples								F_{ST}
		Tro	**Ob**	**Ga**	**Tex**	**Do**	**LT**	**Per**	**Fa**	
*MDH-B**	**75*	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.030
	**100*	1.000	1.000	0.942	1.000	1.000	1.000	1.000	1.000	
	**115*	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000	
N		49	40	52	61	57	56	52	53	
H_e		0.000	0.000	0.112	0.000	0.000	0.000	0.000	0.000	
H_o		0.000	0.000	0.056	0.000	0.000	0.000	0.000	0.000	
*PGM-1**	**86*	0.104	0.000	0.070	0.000	0.020	0.046	0.023	0.009	0.016
	**100*	0.896	0.975	0.910	0.984	0.980	0.918	0.942	0.953	
	**106*	0.000	0.000	0.000	0.008	0.000	0.000	0.035	0.000	
	**114*	0.000	0.025	0.020	0.008	0.000	0.036	0.000	0.038	
N		48	40	50	61	51	55	43	53	
H_e		0.189	0.049	0.168	0.033	0.039	0.155	0.112	0.092	
H_o		0.125	0.050	0.180	0.033	0.039	0.164	0.116	0.094	
*PGM-2**	**90*	0.000	0.000	0.000	0.027	0.000	0.000	0.000	0.000	0.357*
	**95*	0.000	0.058	0.019	0.696	0.536	0.286	0.571	0.500	
	**100*	1.000	0.942	0.972	0.277	0.464	0.714	0.429	0.500	
	**120*	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000	
N		47	26	53	56	28	42	35	46	
H_e		0.000	0.111	0.056	0.442	0.507	0.413	0.497	0.506	
H_o		0.000	0.115	0.057	0.446	0.357	0.381	0.229	0.522	
MULTI-LOCUS										0.247*
P		0.188	0.375	0.375	0.313	0.588	0.438	0.333	0.250	
H_e		0.062 (0.141)	0.150 (0.284)	0.088 (0.150)	0.098 (0.185)	0.148 (0.209)	0.150 (0.194)	0.104 (0.185)	0.066 (0.144)	
H_o		0.054 (0.127)	0.141 (0.279)	0.095 (0.172)	0.093 (0.174)	0.128 (0.190)	0.140 (0.185)	0.085 (0.153)	0.062 (0.143)	
MNA		1.19	1.38	1.56	1.56	1.59	1.81	1.67	1.44	

Following loci are fixed for the same allele: *AK*, CK-A*, FH*, LDH-C**

Appendix 3.2. *Pomatoschistus microps*: partial cytochrome b sequences of the various haplotypes with EMBL accession numbers. Only variable sites and their positions are represented. H1 is employed as reference sample. 'H' refers to Haplotype.

Accession number	H	0 1 9	0 2 0	0 3 1	0 3 3	0 3 9	0 4 5	0 4 8	0 5 4	0 8 4	0 8 7	1 1 1	1 3 8	1 4 1	1 7 7	1 8 3	1 8 9	1 9 5	1 9 9	2 0 1	2 0 1	2 1 6	2 1 6	2 4 9	2 4 0	2 4 3	2 4 7	2 5 2	2 5 8	2 6 7	2 6 8	2 7 0	2 7 3	2 7 4	2 7 5	2 7 6
AJ550471	H1	A	T	T	G	C	C	A	A	T	A	C	C	G	T	T	C	A	C	G	A	T	G	T	A	G	G	T	G	A	G	C	G	C	C
AJ550472	H2	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ550473	H3	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	C	.	.
AJ550481	H4	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.
AJ550482	H5	.	C	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.	G	.	G	.	.	.
AJ550507	H6	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
AJ550480	H7	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.
AJ550479	H8	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.
AJ550486	H9	.	.	.	.	.	.	.	G	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.
AJ550504	H10	.	.	C	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
AJ550495	H11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	G	.
AJ550476	H12	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	A	.	.	.
AJ550475	H13	.	.	.	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	A	.	.	.
AJ550477	H14	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	.	.
AJ550478	H15	.	.	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	A	.	.	.
AJ550487	H16	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	G	.	C	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.
AJ550491	H17	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	G	.	.	.	.	.	.	G	.	.	.
AJ550485	H18	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	.	.	C	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.
AJ550501	H19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
AJ550502	H20	.	.	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
AJ550493	H21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
AJ550499	H22	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ550489	H23	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.	.	.	.	.	G	.
AJ550490	H24	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.	.	.	G	.	.	T
AJ550488	H25	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.
AJ550494	H26	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ550484	H27	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.
AJ550474	H28	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	G	.	.	.	A	.	G	.	.	.	.	.	.	A	.	.	.
AJ550506	H29	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
AJ550492	H30	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.	.
AJ550500	H31	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
AJ550505	H32	.	.	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
AJ550503	H33	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	.	.	.
AJ550498	H34	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ550508	H35	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	G	.	.	.
AJ550497	H36	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ550483	H37	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	A	.	.	.	.	.	G	.	.	.
AJ550496	H38	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Appendix 3.3*: Pomatoschistus microps*: distribution of haplotypes per sampling area. For sample abbreviations see Table 3.1. 'H' refers to the haplotype.

H	Tro	Ob	Ga	Tex	Da	Do	Bdu	Ply	LT	Per	Vac	Fa
H1				1	20	2	4	6	19			1
H2						1						
H3						1		1	4			1
H4		13	19	20		10	17	2				
H5		1										
H6					1		2		2			11
H7												1
H8												1
H9												1
H10												1
H11												2
H12										7	8	
H13										1	1	
H14	27					2				10	10	
H15										1	1	
H16			1									
H17			7									
H18								1				
H19								3				
H20									3			
H21									1			
H22									2			
H23								1				
H24				1								
H25												1
H26												1
H27							2					
H28										1		
H29												2
H30							1					
H31												1
H32												2
H33									1			
H34												1
H35												1
H36												1
H37				3								
H38					1							
Total	27	14	27	25	22	16	26	14	32	20	20	29

CHAPTER IV

SMALL-SCALE GENETIC VARIATION IN *POMATOSCHISTUS MINUTUS* AND *P. LOZANOI* ON THE BELGIAN CONTINENTAL SHELF AND THE SCHELDE ESTUARY

PAPER IV

Small-scale genetic variation in relation to environmental heterogeneity in the marine gobies *Pomatoschistus minutus and P. lozanoi* (Teleostei)

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ABSTRACT

Genetic variation was assessed at 14 allozyme loci in estuarine, coastal and offshore samples of lozano's goby, *Pomatoschistus lozanoi* and the sand goby, *P. minutus*. Samples were taken in habitats on the Belgian Continental Shelf and in the Schelde estuary subject to various degrees of environmental heterogeneity in order to assess (1) whether any correlation could be found between environmental heterogeneity and genetic diversity and (2) if so, if this could be linked to fitness parameters. No significant differences in levels of genetic diversity were recorded between estuarine, coastal and offshore samples in either species. Homozygotes showed a slightly lower condition factor than heterozygotes in both species but the difference was not significant. A temporally stable gradient in allele frequencies at the two-allele locus *GPI-A** was observed in *P. lozanoi,* differentiating the samples in an estuarine, coastal and offshore group. We suggest that these differences might be maintained by balancing selection at locus *GPI-A**.

Keywords: *allozymes, environmental heterogeneity, gobies, Belgian Continental Shelf*

INTRODUCTION

The adaptive significance of allozymatic polymorphisms has been the subject of a heated debate for years, until it waned in the early nineties with the advance of DNA techniques (Hey, 1999). Allozyme-based population surveys had revealed high levels of polymorphism with no visible effects on the phenotype of the organisms, suggesting that the majority of the variation found at the molecular level was selectively neutral (Kimura, 1979). DNA data at first seemed to confirm this hypothesis, with non-coding DNA evolving much faster and containing a higher amount of variability than coding regions. But while this neutral theory of evolution continues to provide a useful null hypothesis against which to test population genetic differentiation, genetic studies of natural populations have yielded convincing evidence that a number of polymorphisms is probably maintained by natural selection at the level of allozymes (Eanes, 2002 and references herein), as well as DNA markers (Ballard & Kreitman, 1995). Both large-scale and small-scale gradients in allele frequencies at several allozyme loci have been reported for a wide range of organisms (Oakeshott et al, 1982; Johannesson *et al*, 1995; Maes & Volckaert, 2002), suggesting environmental selection pressure on the loci under study, with variability in temperature and (for marine species) salinity being considered as main selective agents. In some cases this was attributed to differential performance of the enzyme products of distinct alleles under different environmental conditions (Watt, 1983; Zera, 1987). A correlation between the level of genetic variation (heterozygosity) and the degree of heterogeneity in the habitat of the species has also been documented (Powell, 1971). It has been argued that this may be maintained either by diversifying selection in a heterogeneous environment (Levinton, 1973) or else heterozygote advantage (Mitton, 1997).

Gobies belonging to the genus *Pomatoschistus* (Gill) have been widely used in ecological, evolutionary and behavioural studies (Fonds, 1973; Jones *et al*, 2001b). An electrophoretic survey among Atlantic and Mediterranean members of the genus *Pomatoschistus* species occurring in habitats with a different degree of heterogeneity showed a positive correlation between the level of genetic variation among species and the degree of heterogeneity of the habitat in which each species preferentially occurs (Walllis & Beardmore, 1984a). For example, *Pomatoschistus lozanoi* (de Buen, 1923), which has in general a rather neritic distribution (Fonds, 1973), showed significantly lower levels of polymorphism and heterozygosity than its estuarine-marine relative *P. minutus*. However, it was unclear whether this could be explained by either heterozygote advantage or else

diversifying selection. If heterozygosity is advantageous *per se*, this implies that the level of within-species heterozygosity should be positively correlated with some fitness parameter of the fish (Thelen & Allendorf, 2001). If, on the contrary, habitat heterogeneity promotes overall levels of polymorphism, then we expect populations of a species occurring in a heterogeneous environment showing higher levels of genetic diversity than those inhabiting more stable environments (Powell, 1971). Alternatively, a single locus under selection might show differences in allele frequencies as expected under the multiple niche model of balancing selection (Levene, 1953), when comparing populations across a heterogeneous environment subject to differential selection pressure. Such a heterogeneous environment exists on the Belgian Continental Shelf (BCS), situated in the Southern Bight of the North Sea. The BCS is characterized by a system of several groups of sand banks alternated with gullies, which are continuously being swept by strong tidal currents. A long-shore and inshore/offshore gradient under the influence of the highly turbid and heterogeneous Schelde estuary is reflected in a number of abiotic factors such as temperature, salinity, turbidity and nutrient concentrations (Nihoul & Hecq, 1984). Consequently, biotic factors such as composition and diversity of marine organisms vary across the BCS (Vanaverbeke *et al*, 2000).

In this study we present the results of an allozyme survey of two marine gobies, *Pomatoschistus lozanoi* (de Buen, 1923) and *Pomatoschistus minutus* (Pallas, 1770), carried out on the Belgian Continental Shelf (BCS) and in the Schelde estuary, for assessing possible effects of natural selection on the maintenance of genetic diversity within two species occurring in a gradient of environmental heterogeneity. We aim to address following questions: (1) does any difference in the degree of genetic variation exist within *P. minutus* and *P. lozanoi* collected in various habitats on the Belgian Continental Shelf? (2) can any correlation be found between genetic variation and fitness parameters? and (3) are there any differences in allele frequencies at individual loci in relation to environmental variables?

MATERIALS AND METHODS

Sampling

Sampling on the Belgian Continental Shelf was carried out with R/V 'Belgica' along an inshore/offshore gradient on the Coastal Banks (Stroombank), Flemish Banks (Kwintebank) as well

as offshore banks (Bligh Bank, Noordhinder and Oosthinder). In addition, a number of governmental monitoring stations (MUMM stations) on the Belgian Continental Shelf were trawled. Fish were caught with a beam trawl (width: 3 m) and an outer net mesh size of 20 mm stretched and a cod end mesh size of 12 mm. The samples from the Schelde estuary were taken in Doel near the city of Antwerp at the cooling water intake of the nuclear power plant (Fig.4.1). Mesh size of the nets used was 4 mm. Sampling stations are grouped by area: the estuary, the inshore sandbanks along the Belgian coast and the offshore sandbanks (Table 4.1.1). For comparison a sample of *P. lozanoi* and *P. minutus* caught at the Frisian Front (north of the Wadden Sea) was subjected to allozyme electrophoresis as well.

Table 4.1.1. Sampling sites on the sand banks of the Belgian Continental Shelf with coordinates and numbers of fish examined (N).

Habitat	Sampling site	Code	Period	Longitude	Latitude	N (*P. minutus*)	N (*P. lozanoi*)
Estuary	Doel	D3	March 1998	51° 19' 30 N	04° 16' 00 E	-	34
	Doel	D10	Oct. 1998	51° 19' 30 N	04° 16' 00 E	71	-
Coast	Kwintebank	Ki2	Feb. 1997	51° 17' 61 N	02° 38' 92 E	68	-
	Stroombank	Sb	Feb. 1997	51° 13' 80 N	02° 52' 42 E	83	-
	Kwintebank	Ki10	Oct. 1997	51° 15' 36 N	02° 38' 34 E	161	42
	MUMM 780 (Raan)	Ra	March 1997	51° 28' 05 N	03° 03' 69 E	50	-
	MUMM 702 (Zeebrugge)	Ze	Oct. 1997	51° 22' 63 N	03° 18' 68 E	-	78
	MUMM 790 (Ostend)	Os	Oct. 1997	51° 16' 87 N	02° 51' 13 E	52	50
	MUMM 120	Ht	Oct. 1998	51° 11' 10 N	02° 47' 07 E	35	-
	GC1	Gc	Feb.1999	51° 34' 40 N	03° 24' 80 E	-	23
	MUMM 250	Gc	Feb. 1999	51° 31' 00 N	03° 19' 00 E	-	13
Offshore	Oosthinder	Oh	Oct. 1997	51° 30' 30 N	02° 38' 32 E	51	31
	Bligh Bank	Bli	Feb. 1998	51° 33' 37 N	02° 44' 16 E	28	60
	Noordhinder	Nh	Oct. 1998	51° 39' 50 N	02° 36' 14 E	-	46
Wadden Sea	Frisian Front	FF	Dec. 1998	53° 30' 00 N	04 °00' 00 E	38	31

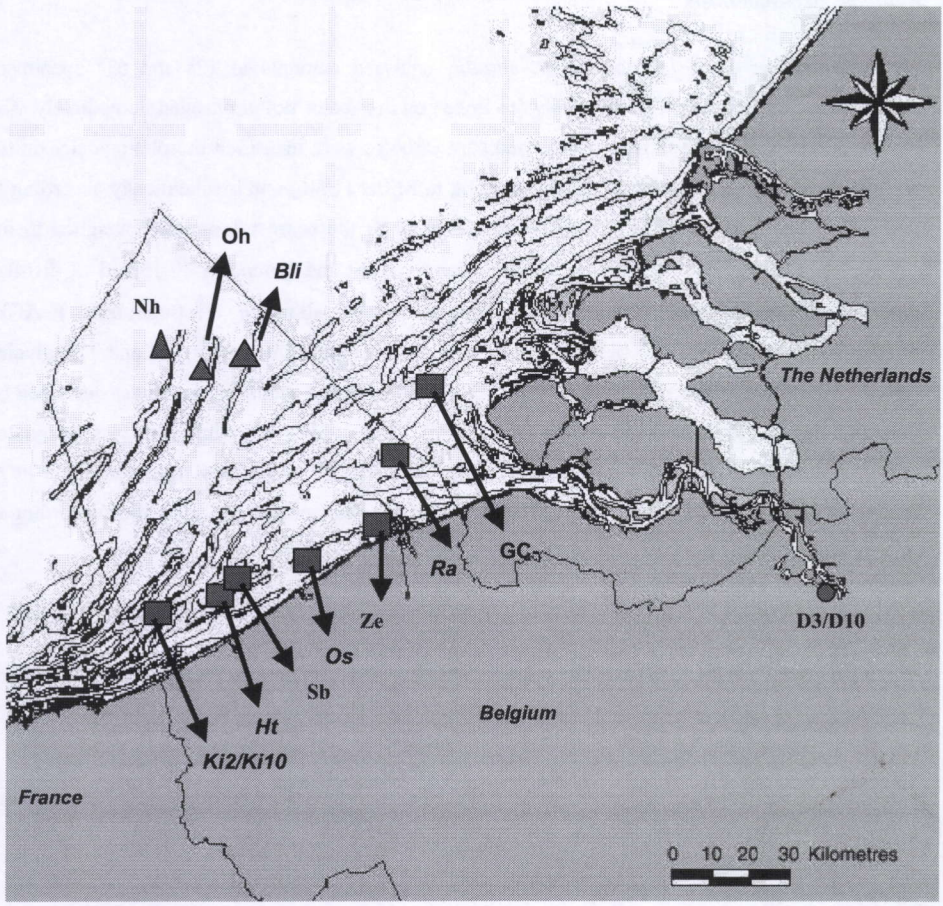


Figure 4.1.1. Sampling locations and codes of the sampling sites of *Pomatoschistus minutus* and *P. lozanoi* on the Belgian Continental Shelf and in the Schelde estuary. Triangles: offshore samples; Squares: coastal samples; Circle: estuarine sample. For the abbreviations, see Table 4.1. The Frisian Front is located north in the Wadden Sea and not indicated on the map.

Allozyme electrophoresis

The total number of *P. lozanoi* and *P. minutus* analysed amounts to 408 and 637 specimens respectively (Table 4.1.1). The samples were frozen on dry ice or liquid nitrogen immediately after capture and kept in a -80°C freezer until analysis. Gobies were identified morphologically on the basis of the dermal papillae of the head according to Miller (1986) and biochemically according to Wallis and Beardmore (1984a,b). Fish were measured to the nearest 1 mm and weighed to the nearest 1 mg. Liver, eye and muscle tissues were dissected and ground in 50-150 µl of distilled water. The samples were subjected to cellulose acetate allozyme electrophoresis (CAGE) (Richardson *et al,* 1986). Two buffer systems were used: Tris-maleate (pH = 7.8) and Tris-glycine (pH = 8.8) following Hebert & Beaton (1989). Loci were stained according to recipes described by Hebert & Beaton (1989) and Richardson *et al* (1986). The slowest migrating locus was designated 1 or A according to the nomenclature of Shaklee *et al* (1990). The most common allele in *P. minutus* at each locus was called '100' and other alleles in either species were classified according to their mobility relative to allele 100 of the locus under study.

Table 4.1.2: Summary of the 14 loci examined including the enzyme code (E.C.) and the tissue employed. M = muscle, E = eye.

Enzyme	Locus (tissue)
Adenylate Kinase kinase (AK) [EC 2.7.4.3]	***AK**** **(M)**
Aspartate aminotransferase (sAAT) [EC 2.6.1.1]	*AAT** (M)
Creatine Kinase kinase (CK) [EC 2.7.3.2]	*CK-A** (M)
Fumarate hydratase (FH) [EC 4.2.1.2]	*FH** (M)
Glucose-6-phosphate isomerase (GPI) [EC 5.3.1.9]	*GPI-A** (M,E), *GPI-B** (M, E)
Isocitrate dehydrogenase (IDHP) [EC 1.1.1.42]	*IDHP** (M)
L-Lactate dehydrogenase (LDH) [EC 1.1.1.27]	*LDH-A** (M), *LDH-B** (M), *LDH-C*** (E)
Malate dehydrogenase (MDH) [EC 1.1.1.37]	*MDH-A** (M), *MDH-B** (M)
Phosphoglucomutase (PGM) [EC 5.4.2.2]	*PGM-1** (M), *PGM-2** (M)

Data analysis

The condition factor (CF), expressed as Weight (in grams) x 100/ Length ^{3} (in cm) is an indicator of metabolic efficiency, as fish with a larger condition factor allocate more energy to growth than to metabolic needs (Danzmann *et al*, 1988). It was computed for each individual fish and a one-way analysis of variance (ANOVA) was performed with the software package STATISTICA version 6.0 (Statsoft 2001), in order to assess if condition differed between sampling sites.

Genetic diversity was estimated as observed and unbiased expected heterozygosity (Nei, 1978) and calculated in GENETIX version 4.0 (Belkhir *et al,* 2001). A locus was considered polymorphic when the frequency of its most common allele did not exceed 0.99 in at least one population. Linkage disequilibrium and Hardy-Weinberg equilibrium were calculated and tested for significance in GENEPOP version 3.1 (Raymond & Rousset, 1995). Significance levels of Hardy-Weinberg equilibrium and linkage disequilibrium were adjusted with a sequential Bonferroni test in order to correct for multiple testing (Rice, 1989). Pairwise F_{ST} values (Weir & Cockerham, 1984) were calculated in GENETIX and significance was assessed with permutation tests (1000 replicates). Differences in allele frequencies between samples were tested in GENEPOP. Multidimensional scaling was applied on a matrix of pair-wise F_{ST} values in order to assess whether any geographical clustering of the samples could be found in STATISTICA. Individual heterozygosity was calculated as the number of polymorphic loci per individual (Thelen & Allendorf, 2001), and a linear regression was computed between the log-transformed condition factor and individual heterozygosity. Samples were grouped into 'coastal', 'estuarine 'and 'offshore' habitats in order to assess whether any significant differences in the level of heterozygosity could be found between habitats. A Kruskall Wallis ANOVA was performed with the number of polymorphic loci as dependent variable and the sampling group as grouping variable.

RESULTS

Nine enzyme systems, corresponding to fourteen enzyme loci, were found suitable for cellulose acetate allozyme electrophoresis (Table 4.1.2). The allele frequencies with observed and expected heterozygosity are listed in Appendix 4.1.1 (*P. minutus*) and Appendix 4.1.2 (*P. lozanoi*). An *a priori* exact test for allelic homogeneity (Raymond and Rousset, 1995) revealed no differentiation

between *P. lozanoi* caught at the adjacent sampling stations GC-1 and 250 (Fig.4.1.1). They were subsequently pooled to increase the sample size and recoded as Gc.

Genetic diversity

Pomatoschistus lozanoi

After sequential Bonferroni correction all samples of *P. lozanoi* were in Hardy-Weinberg equilibrium and no linkage disequilibrium was observed. The values for the observed and the expected heterozygosity, the mean number of alleles and the proportion of polymorphic loci differ slightly across all samples from the Belgian coast, ranging from 0.038 to 0.056. Apparently, this is not correlated with the habitat: observed heterozygosity is highest in the offshore site Bligh bank (H_o = 0.052) and similar to the values for the estuarine sample from Doel (H_o = 0.042) and Oosthinder (H_o = 0.046), while the lowest heterzogyosity is found in the coastal site of Zeebrugge (H_o = 0.034) (Table 4.1.3a). When samples were pooled according to the various habitats (estuarine, coastal and offshore), no significant differences in levels of heterozygosity were found either (Kruskall Wallis ANOVA: P = 0.457).

Table 4.1.3a. *Pomatoschistus lozanoi*: Genetic diversity expressed as unbiased expected heterozygosity (H_e), observed heterozygosity (H_o), percentage of polymorphic loci, mean number of alleles per locus (MNA). Average standard length in mm (SL) and condition factor (CF) per sampling site with the standard deviations between parentheses are included.

Habitat	Site	H_e	H_o	P	MNA	SL	CF
Estuary	D3	0.050 (0.085)	0.042 (0.084)	0.357	1.50	40.78 (4.68)	1.189 (0.116)
Coastal	Gc	0.054 (0.140)	0.035 (0.099)	0.286	1.50	45.75 (4.31)	1.117 (0.075)
	Ze	0.038 (0.093)	0.034 (0.081)	0.214	1.43	35.15 (6.63)	0.927 (0.092)
	Ki10	0.047 (0.099)	0.048 (0.099)	0.357	1.43	38.88 (6.69)	1.049 (0.154)
	Os	0.039 (0.105)	0.037 (0.099)	0.286	1.43	34.45 (6.15)	0.957 (0.125)
Offshore	Bli	0.056 (0.136)	0.052 (0.129)	0.286	1.57	44.05 (6.20)	0.956 (0.197)
	Nh	0.048 (0.125)	0.040 (0.096)	0.429	1.50	39.48 (4.91)	0.973 (0.133)
	Oh	0.043 (0.134)	0.046 (0.143)	0.143	1.14	40.57 (7.18)	0.979 (0.188)
Wadden Sea	FF	0.045 (0.121)	0.038 (0.095)	0.214	1.29	40.54 (5.14)	0.890 (0.095)

Pomatoschistus minutus

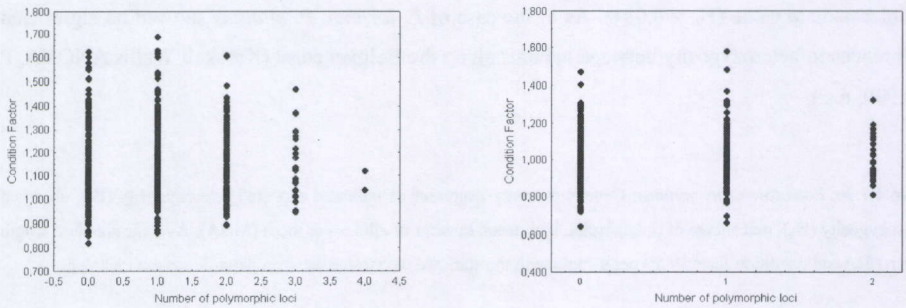
Locus *LDH-C** in *P. minutus* showed a significant heterozygote deficit across all samples, with the exception of Stroombank and MUMM 120 (Ht). All other loci were in HW equilibrium in all samples. No linkage disequilibrium was observed after sequential Bonferroni correction. Values for heterozygosity, proportion of polymorphic loci and mean number of alleles per locus in the *P. minutus* samples collected along the Belgian coast differed, but no correlation between genetic diversity with habitat was apparent. For example, observed heterozygosity ranges from 0.065 (MUMM 120) to 0.104 (Stroombank) (Table 4.1.3.b), while the estuarine sample from Doel showed an intermediate value (H_o = 0.084). As in the case of *P. lozanoi, P. minutus* showed no significant differences in heterozygosity between habitats along the Belgian coast (Kruskall Wallis ANOVA, P = 0.900, n.s.).

Table 4.1.3b. *Pomatoschistus minutus*: Genetic diversity expressed as unbiased expected heterozygosity (H_e), observed heterozygosity (H_o), percentage of polymorphic loci, mean number of alleles per locus (MNA). Average standard length in mm (SL) and condition factor (CF) per sample with the standard deviations between parentheses are included.

Habitat	Site	H_e	H_o	P	MNA	SL	CF
Estuary	D10	0.099 (0.185)	0.084 (0.160)	0.357	2.07	47.99 (8.09)	1.351 (0.087)
Coast	Ki2	0.099 (0.210)	0.086 (0.179)	0.214	1.64	50.24 (9.06)	1.159 (0.166)
	Sb	0.102 (0.204)	0.104 (0.220)	0.286	1.50	44.13 (7.88)	1.138 (0.200)
	Ra	0.110 (0.208)	0.096 (0.189)	0.429	1.71	44.71 (6.04)	1.124 (0.108)
	Ki10	0.110 (0.210)	0.093 (0.168)	0.286	2.14	50.24 (9.06)	1.155 (0.125)
	Ht	0.084 (0.167)	0.065 (0.120)	0.357	1.57	49.71 (8.75)	1.269 (0.117)
	Os	0.111 (0.219)	0.086 (0.170)	0.286	1.79	45.71 (8.52)	1.098 (0.148)
Offshore	Bli	0.102 (0.183)	0.067 (0.110)	0.357	1.64	45.00 (5.47)	1.006 (0.127)
	Oh	0.110 (0.208)	0.084 (0.168)	0.429	1.86	45.18 (8.47)	1.102 (0.120)
Wadden Sea	FF	0.108 (0.212)	0.064 (0.113)	0.357	1.64	41.50 (7.86)	0.915 (0.094)

Genetic diversity and condition factor

In both species the condition factor was slightly higher for heterozygotes than for homozygotes, but the difference was not significant (*P. lozanoi*: 1.02 vs. 0.99, ANOVA, $P = 0.134$; *P. minutus*: 1.121 vs 1.134, ANOVA, $P = 0.498$). A linear regression between condition factor and number of polymorphic loci was not significant either (*P. min:* $R^2 = 0.042$, $P = 0.310$; *P. loz*: $R^2 = 0.072$, $P = 0.144$) (Fig. 4.1.2 a and b).



(a) (b)

Figure 4.1.2. Relationship between condition factor and number of polymorphic loci. a) *P. minutus*, b) *P. lozanoi*

Genetic differentiation

P. minutus differentiated between sampling sites at *LDH-C** (Table 4.1.4) but this locus was excluded from the overall F_{ST} analysis due to the large deviations from HW equilibrium. Without locus *LDH-C** only the pair-wise F_{ST} value between Doel and the Frisian Front turned out to be significant (pair-wise $F_{ST} = 0.083$, $P = 0.001$).

Table 4.1.4. Allele frequency differences between samples significant at the 0.01 level.

P. minutus			*P. lozanoi*		
Locus	**Sample 1**	**Sample 2**	**Locus**	**Sample 1**	**Sample 2**
*LDH-C**	Ost	Sb	*GPI-A**	Ki10	Nh
	Ost	Ht		Ki10	Oh
	Oh	Ki2		Ki10	Bli
	Oh	Sb		Ze	Nh
	Ra	Ht		Ze	Oh
	Ki2	Ht		Ze	Bli
	Sb	Ht		Os	Bli
				Nh	D3
				Oh	D3
				Bli	D3
				FF	D3

The multi-locus F_{ST} across all samples and loci (without *LDH-C**) from the Belgian Continental Shelf for *P. minutus* amounted to 0.0058. In *P. lozanoi*, pair-wise comparisons were significant for allele frequency differences at locus *GPI-A** (Table 4.1.4). Multi-locus F_{ST} value across all samples with *GPI-A** included amounted to 0.029, but was only -0.002 without locus *GPI-A**.

The pair-wise differences in allele frequencies at locus *GPI-A** for *P. lozanoi* clearly differentiated the samples in an estuarine, coastal and offshore group (Fig. 4.1.3). The Frisian Front groups together with the offshore samples from the Belgian coast.

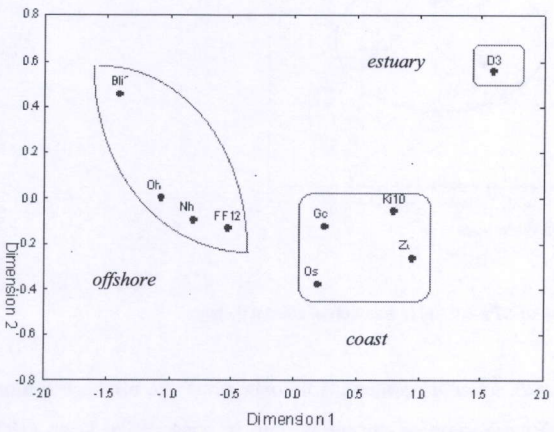


Figure 4.1.3. *P. lozanoi*: Multidimensional Scaling based on pair-wise F_{ST} values (Weir & Cockerham, 1984) at locus *GPI-A**. Stress value = 0.0085.

Clinal variation at locus *GPI-A of the alleles *GPI-A*100* and *GPI-A*113* in *Pomatoschistus lozanoi***

In *P. lozanoi* locus *GPI-A** presents two alleles occurring in appreciable frequencies, *GPI-A*100* and *GPI-A*113* (Appendix 4.1.2). When plotting the frequency of allele *GPI-A*100* in *P. lozanoi* against the distance from the coast the occurrence increases on an inshore-offshore axis, with the lowest frequencies in Doel and the highest at the Hinderbanken (Fig. 4.1.4). A Spearman Rank correlation showed a significant relationship between coastal distance and the frequency of *GPI-A*100* ($P < 0.05$). In Doel no *GPI-A*100/100* homozygotes and only few *GPI-A*100/113* heterozygotes were recorded. In *P. minutus* this locus shows minimal polymorphism and no gradient in allele frequencies is observed.

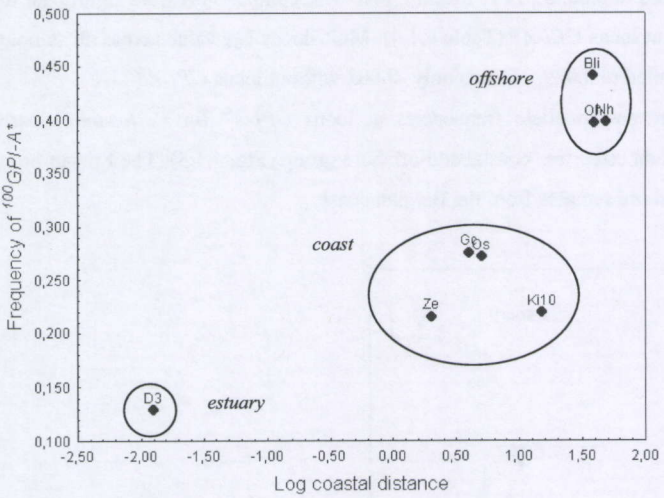


Figure 4.1.4. *Pomatoschistus lozanoi*: Frequency of *GPI-A* 100* in function of coastal distance.

The differences at the *GPI-A** locus for *P. lozanoi* seemed stable over time: the offshore stations Noordhinder (Nh) and Oosthinder (Oh) were sampled during autumn in consecutive years (1998 and 1997) while the Bligh Bank (Bli) was sampled in February 1998, but the allele frequencies remained similar (Fig. 4.1.4). The same is true for the coastal samples: GC1-250 (Gc) was sampled

in February 1999 while Kwintebank (Ki10) and MUMM station 702 (Ze) were sampled during autumn 1997. Assuming that the gradient in allele frequencies at *GPI-A** may be maintained by natural selection, this implies that the respective genotypes must perform differently in their respective habitat (estuarine, coastal and offshore). Thus, in order to assess whether any correlation existed between fitness and the occurrence of the respective alleles, individuals were divided into three groups according to their genotype at *GPI-A** (*GPI-A* 100/100, GPI-A*100/113* and *GPI-A*113/113*) by sampling site.

Table 4.1.5. *Pomatoschistus lozanoi*: Frequency of alleles at *GPI-A** per sampling site and average value for the Condition Factor (CF) per genotype.

Habitat	Site	*GPI-A*100*	*GPI-A*113*	Genotype	CF
Estuary	D3	0.130	0.870	*GPI-A*100/100*	-
				*GPI-A*100/113*	1.171
				*GPI-A*113/113*	1.192
Coast	Os	0.272	0.728	*GPI-A*100/100*	0.889
				*GPI-A*100/113*	0.979
				*GPI-A*113/113*	0.966
	Ze	0.215	0.785	*GPI-A*100/100*	0.881
				*GPI-A*100/113*	0.940
				*GPI-A*113/113*	0.929
	Gc2	0.276	0.724	*GPI-A*100/100*	1.086
				*GPI-A*100/113*	1.119
				GPI-A 113/113*	1.140
	Ki10	0.220	0.781	*GPI-A*100/100*	0.921
				*GPI-A*100/113*	1.045
				*GPI-A*113/113*	1.033
Offshore	Bli	0.441	0.559	*GPI-A*100/100*	0.994
				*GPI-A*100/113*	0.943
				*GPI-A*113/113*	0.949
	Oh	0.397	0.603	*GPI-A*100/100*	0.986
				*GPI-A*100/113*	0.977
				*GPI-A*113/113*	0.985
	Nh	0.398	0.602	*GPI-A*100/100*	0.969
				*GPI-A*100/113*	1.028
				*GPI-A*113/113*	0.922

Although all ANOVA test results for condition factor by genotype were non-significant, condition factor was always slightly lower for individuals homozygote for *GPI-A*100* in the coastal samples, while fish homozygous for *GPI-A*113* had the lowest condition factor in the offshore samples (Table 4.1.5). The only exception is the Oh sample, with almost identical values for the three genotypes.

LDH-C** in *P. minutus

Pomatoschistus minutus was differentiated between sampling sites at locus *LDH-C**, but no gradient in allele frequencies was observed. In order to assess whether selection against heterozygotes might be invoked for explaining the observed heterozygote deficit at this locus, condition factors were compared between (1) homozygotes and heterozygotes at locus *LDH-C** and (2) the various genotypes at *LDH-C** (*LDH-C*100/100, LDH-C*100/107, LDH-C*107/107, LDH-C*100/115, LDH-C*107/115* and *LDH-C*115/115* respectively), but no significant effects were recorded (ANOVA, $P > 0.05$).

DISCUSSION

Small-scale genetic variation in relation to environmental heterogeneity

The allozyme data show a lower degree in heterozygosity in *Pomatoschistus lozanoi* (H_o: 0.042 - 0.052; H_e: 0.038-0.054) than in *P. minutus* (H_o: 0.064 to 0.104; H_e: 0.084-0.111). This confirms the results of Wallis & Beardmore (1984b), who reported comparable expected heterozygosities for *P. lozanoi* ($H_e = 0.054$) and *P. minutus* ($H_e = 0.078$) in a study based on 31 loci. Thus, observed and expected heterzoygosity for *P. minutus* is about twice as high as in *P. lozanoi*. This interspecific difference in genetic diversity has been linked to the degree of heterogeneity of the environment in which both species are generally found (Wallis and Beardmore, 1984b). *P. minutus* is more coastal-estuarine while *P. lozanoi* has a rather neritic distribution (Fonds, 1973). However, an argument against this could be complete the overlap in the distributional range of these species on the Belgian Continental Shelf (Table 4.1.1). Other studies point also at high numbers of the putatively more neritic *P. lozanoi* in the estuarine environment (Claridge *et al*, 1985; Arruda *et al*, 1993; Maes *et al*,

1998a). In our study, no evidence at all was found for increased levels of heterozygosity in either species for individuals occurring in estuarine (heterogeneous) versus marine (more stable) habitats, as we would expect if habitat heterogeneity promotes genetic diversity. Sorice & Caputo (1999) could not find any evidence for the adaptation arguments either, as no particular ecological differences were found between highly and less variable species in their survey of Mediterranean goby species. They noticed that highly polymorphic Mediterranean goby species were also karyologically highly variable and suggested genomic constraints in chromosome variation as an alternative hypothesis for explaining different levels of polymorphism between goby species. However, Webb (1980) reported no intra- or interindividual variation in chromosome morphology within *P. minutus* or *P. lozanoi,* excluding this as a hypothesis for these species. An alternative explanation for the observed differences in levels of genetic diversity between *P. minutus* and *P. lozanoi* may simply be found in random drift and stochastic events such as bottlenecks or founder effects since the divergence of the two species.

We did observe a slight increase in condition factor for heterozygotes as opposed to homozygotes in both species across all sampling sites, which could point to heterozygote advantage. However, considering that the difference was far from significant, no firm conclusions can be drawn from our dataset. Heterozygosity-fitness correlations have been recorded for several fish species (Thelen & Allendorf, 2001 and references herein), but numerous other studies did not find any correlation at all (e.g. Houle, 1989; Savolainen & Hedrick, 1995). As pointed out by David (1998), a correlation between heterozygosity and fitness seems to be by no means universal and a publication bias towards significant results probably exists. Alternatively, other parameters than condition factor might be better suited for estimating fitness in *Pomatoschistus* sp.

Small-scale population differentiation in *Pomatoschistus* sp. or natural selection on specific loci?

In each species we recorded a significant amount of population differentiation based on allele frequency differences at a single locus: *GPI-A** in *P. lozanoi* and *LDH-C** in *P. minutus*. Loci *PGM-1** and *PGM-2** are also highly polymorphic in either species but no differentiation was recorded. The fact that genetic drift and gene flow should affect all polymorphic loci similarly (Pogson *et al*, 1995) points to some other underlying mechanism than reproductive isolation for

explaining the observed differentiation. Moreover, considering (1) the extended pelagic phase, which lasts for at least one month (Fonds, 1973) and (2) the extensive currents in the area, a large amount of gene flow between *Pomatoschistus* sp. from the Southern Bight is not unlikely.

GPI-A in Pomatoschistus lozanoi*

The fact that the reference sample from the Frisian Front, an offshore location, groups together with the offshore Belgian samples, based on its allele frequencies at locus *GPI-A** (Fig. 4.1.2), supports the hypothesis that some environmental factor might be responsible for the observed allele frequencies. Christiansen & Frydenberg (1974) reported a similar geographic cline in allele frequencies at two esterase loci in eelpout, while frequencies at other polymorphic loci remained constant, and suggested that these clines were maintained by selective pressure.

Levene ´s model (1953) of balancing selection at a two-allele locus states that both alleles may be maintained in the population by natural selection when selection pressure varies between niches, resulting in a higher fitness of the alternative homozygote genotypes in the various habitats. If we apply this to the Lozano´s gobies investigated on the Belgian Continental Shelf and in the Schelde estuary, this would mean that the fish carrying allele *GPI-A*100* may perform less in estuarine conditions whereas individuals possessing allele *GPI-A*113* might be less adapted to the marine environment. The slightly lower values of the condition factor for the respective genotypes in the various habitats may lend some support to the hypothesis of balancing selection. However, considering that these differences in condition factor were not significant, and that locus *GPI-A** was in Hardy-Weinberg equilibrium, any selection pressure on this locus is not likely to be strong. Moreover, *Pomatoschistus* sp. is heavily subjected to parasitism, which also influences the condition of the fish (Claridge *et al*, 1985).

Although a correlation between allele frequencies at locus *GPI-A** and temperature in fish (including gobies) and other organisms has been reported (Watt, 1983; Al-Hassan *et al*, 1987; Zera *et al*, 1987) there was no such evidence in this study. The sites were sampled in different seasons, coastal sites showing higher variance in temperature than offshore sites, yet allele frequencies at *GPI-A** within groups remained similar. Oxygen concentration at the sampling sites did not show any trend that might be linked to the differences at *GPI-A**. Salinity, which increases with increasing distance from the Schelde estuary, yielded a weak but significantly positive linear correlation with the frequency of *GPI-A*100* ($R^2 = 0.52$, $P < 0.04$). De Wolf *et al* (2001) reported

allozymatic differences at the esterase locus between samples of *Littorina littorea* in the Schelde estuary and adjacent areas and suggested either the influence of salinity or anthropogenic effects as responsible agents for the differentiation. It is tempting to point to the salinity gradient for explaining the observed differences at *GPI-A**, but any other factor increasing with increasing distance to the coast would show a similar correlation. For example, the Schelde estuary is a heavily polluted area while the level of pollution decreases towards the offshore areas. The water flowing through the English Channel towards the Southern Bight is relatively clean Atlantic water. A number of studies have revealed differential mortality of distinct genotypes when exposed to heavy metals, suggesting they could be useful markers for assessing levels of pollution (Ben-Shlomo & Nevo, 1988). For example, Nevo *et al* (1984) and Newman & Jagoe (1998) found evidence for an allele shift at the *GPI** loci in shrimps and fish respectively, when exposed to mercury. However, in order to prove an association between the observed *GPI-A** genotypes and the level of pollution, this would require detailed biochemical experiments

LDH-C in Pomatoschistus minutus*

The only significant differences in allele frequencies between the *P. minutus* samples were found for *LDH-C**, which showed a consistent heterozygote deficit across almost all *Pomatoschistus minutus* samples. Such a deficit might be attributed to scoring error, null alleles, selection against heterozygotes or the Wahlund effect. Scoring error is ruled out because a very distinct three-allele-polymorphism was recorded at this locus using CAGE, contrary to starch gel electrophoresis (Wallis & Beardmore, 1984a,b; Stefanni, 2000a). If the heterozygote deficit were due to a mixing of subpopulations, we would have expected a similar effect at the other polymorphic loci, which is not the case. Null alleles may be another explanation (Richardson *et al*, 1986). Selective mortality of heterozygotes has often been invoked for the heterozygote deficit observed in marine organisms (Zouros & Foltz, 1984; Kotoulas *et al*, 1995). However, assuming that the condition factor represents some indication of the general fitness of the fish, we did not find any evidence for a reduced fitness in heterozygotes at *LDH-C**.

CONCLUSIONS

Our study did not find any differences in level of genetic variation in *P. minutus* and *P. lozanoi,* which could be linked to the degree of heterogeneity of the habitats where the samples were collected. We can confirm the lower degree of genetic variation in *P. lozanoi* compared to *P. minutus*. However, we cannot support the hypothesis that this difference may be due to their occurrence in habitats subject to a different degree of heterogeneity as *P. minutus* and *P. lozanoi* occur sympatrically throughout the whole sampling range. Heterozygotes revealed a slightly higher condition factor than homozygotes across all habitats, but the difference was not significant. Thus, we cannot demonstrate any firm heterozygote-fitness correlations. In *P. lozanoi* no significant population differentiation was recorded except for a clinal variation in allele frequencies at locus *GPI-A**. We suggest that this is due to the effect of natural selection rather than reproductive isolation. *P. minutus* was significantly differentiated at locus *LDH-C*,* but also showed a heterozygote deficit in most samples for this locus.

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Appendix 4.1.1. *Pomatoschistus minutus*: Allele frequencies per sample with observed (H_o) and unbiased expected heterozygosity (H_e), proportion of polymorhic loci (P), mean number of alleles per locus (MNA); N: number of fish screened per sample. Est: estuarine sample, WS: Wadden Sea.

Locus	Allele	Samples									
		Est	Coast						Offshore		WS
		D10	Ki2	Sb	Ra	Ki10	Ht	Os	Bli	Oh	FF
*AAT**	**90*	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	**100*	0.993	1.000	1.000	0.982	1.000	0.986	1.000	1.000	1.000	1.000
	**150*	0.000	0.000	0.000	0.018	0.000	0.014	0.000	0.000	0.000	0.000
N		71	49	64	28	114	35	47	26	48	23
H_e		0.014	0.000	0.000	0.036	0.000	0.029	0.000	0.000	0.000	0.000
H_o		0.014	0.000	0.000	0.036	0.000	0.029	0.000	0.000	0.000	0.000
*GPI-A**	**90*	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000
	**100*	0.979	1.000	1.000	1.000	0.994	0.971	1.000	1.000	0.990	1.000
	**113*	0.021	0.000	0.000	0.000	0.003	0.029	0.000	0.000	0.010	0.000
N		70	65	66	50	156	34	51	24	50	36
H_e		0.042	0.000	0.000	0.000	0.013	0.058	0.000	0.000	0.020	0.000
H_o		0.043	0.000	0.000	0.000	0.013	0.059	0.000	0.000	0.020	0.000
*GPI-B**	**90*	0.014	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.010	0.000
	**100*	0.971	0.992	0.986	0.960	0.971	1.000	0.990	1.000	0.990	1.000
	**116*	0.014	0.008	0.014	0.020	0.029	0.000	0.010	0.000	0.000	0.000
N		70	65	72	50	157	34	51	24	49	35
H_e		0.056	0.015	0.028	0.078	0.056	0.000	0.020	0.000	0.020	0.000
H_o		0.043	0.015	0.028	0.080	0.057	0.000	0.020	0.000	0.020	0.000
*IDHP**	**90*	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000
	**100*	0.993	1.000	1.000	1.000	0.995	1.000	1.000	0.955	1.000	1.000
	**115*	0.000	0.000	0.000	0.000	0.005	0.000	0.000	0.022	0.000	0.000
N		71	27	49	38	98	35	41	22	35	26
H_e		0.014	0.000	0.000	0.000	0.010	0.000	0.000	0.090	0.000	0.000
H_o		0.014	0.000	0.000	0.000	0.010	0.000	0.000	0.091	0.000	0.000
LDH-A*	**60*	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.010	0.014
	**100*	0.991	0.992	1.000	1.000	0.994	1.000	1.000	1.000	0.990	0.972
	**135*	0.009	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.014
N		57	64	79	50	155	35	51	28	48	36
H_e		0.018	0.016	0.000	0.000	0.013	0.000	0.000	0.000	0.021	0.055
H_o		0.018	0.016	0.000	0.000	0.013	0.000	0.000	0.000	0.021	0.056
*LDH-B**	**95*	0.000	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000
	**100*	1.000	0.992	1.000	0.990	1.000	1.000	0.980	0.964	1.000	1.000
	**112*	0.000	0.008	0.000	0.000	0.000	0.000	0.020	0.036	0.000	0.000
N		66	60	77	49	146	35	51	28	40	27
H_e		0.000	0.017	0.000	0.020	0.000	0.000	0.039	0.070	0.000	0.000
H_o		0.000	0.017	0.000	0.020	0.000	0.000	0.039	0.071	0.000	0.000

Appendix 4.1.1 (continued)

Locus	Allele	Samples									
		Est	Coast						Offshore		WS
		D10	Ki2	Sb	Ra	Ki10	Ht	Os	Bli	Oh	FF
*LDH-C**	**100*	0.734	0.560	0.667	0.649	0.615	0.871	0.578	0.732	0.659	0.542
	**107*	0.156	0.276	0.265	0.192	0.198	0.086	0.167	0.143	0.068	0.188
	**115*	0.110	0.164	0.068	0.160	0.183	0.043	0.255	0.125	0.273	0.271
	*120	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000	0.000	0.000
N		64	58	66	47	144	35	51	28	22	24
H_e		0.428	0.588	0.484	0.522	0.551	0.235	0.578	0.436	0.498	0.611
H_o		0.281	0.483	0.439	0.298	0.403	0.171	0.373	0.214	0.136	0.292
*MDH-A**	**60*	0.000	0.000	0.000	0.000	0.000	0.000	0.010	0.000	0.011	0.000
	**100*	1.000	1.000	0.991	1.000	0.994	1.000	0.990	1.000	0.968	0.986
	**140*	0.000	0.000	0.009	0.000	0.006	0.000	0.000	0.000	0.021	0.014
N		71	61	56	49	156	35	52	28	47	36
H_e		0.000	0.000	0.018	0.000	0.013	0.000	0.019	0.000	0.063	0.028
H_o		0.000	0.000	0.018	0.000	0.013	0.000	0.019	0.000	0.064	0.028
*PGM-1**	**86*	0.123	0.083	0.172	0.133	0.147	0.161	0.122	0.146	0.159	0.135
	**100*	0.862	0.900	0.828	0.847	0.833	0.823	0.857	0.833	0.830	0.838
	**114*	0.015	0.017	0.000	0.020	0.020	0.016	0.020	0.021	0.011	0.027
N		65	60	61	49	153	31	49	24	44	37
He		0.244	0.184	0.287	0.267	0.285	0.302	0.252	0.290	0.290	0.283
Ho		0.215	0.167	0.246	0.245	0.307	0.290	0.184	0.333	0.341	0.307
PGM-2*	**90*	0.078	0.108	0.125	0.132	0.076	0.120	0.138	0.056	0.095	0.083
	**95*	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	**100*	0.588	0.549	0.466	0.434	0.529	0.600	0.466	0.333	0.405	0.292
	**103*	0.029	0.000	0.000	0.000	0.048	0.000	0.017	0.000	0.048	0.000
	**106*	0.275	0.343	0.409	0.434	0.343	0.280	0.379	0.611	0.452	0.625
	**110*	0.020	0.000	0.000	0.000	0.005	0.000	0.000	0.000	0.000	0.000
N		51	51	44	38	105	25	29	9	42	12
He		0.577	0.575	0.607	0.614	0.598	0.558	0.631	0.543	0.628	0.540
Ho		0.549	0.510	0.727	0.658	0.476	0.360	0.552	0.222	0.571	0.250

Loci *FH**, *AK** and *MDH-B** were fixed for the same allele **100*.

Appendix 4.1.2. *Pomatoschistus lozanoi*: Allele frequencies per sample with observed (H_o) and unbiased expected heterozygosity (H_e), proportion of polymorhic loci (P), mean number of alleles per locus (MNA); N: number of fish screened per sample.

Locus	Allele	Samples								
		Est.	Coast				Offshore			WS
		D3	Gc	Ze	Ki10	Os	Bli	Nh	Oh	FF
*AAT**	*90	0.000	0.000	0.000	0.000	0.011	0.000	0.012	0.000	0.000
	*100	0.979	1.000	1.000	1.000	0.989	1.000	0.988	1.000	1.000
	*150	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
N		24	36	62	25	45	51	43	31	16
H_e		0.042	0.000	0.000	0.000	0.022	0.000	0.023	0.000	0.000
H_o		0.042	0.000	0.000	0.000	0.022	0.000	0.023	0.000	0.000
*CK-A**	*107	1.000	1.000	1.000	1.000	1.000	1.000	0.988	1.000	1.000
	*111	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000
N		32	36	75	42	52	60	43	34	31
H_e		0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000
H_o		0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000
*GPI-A**	*90	0.000	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	*100	0.140	0.430	0.217	0.220	0.271	0.440	0.378	0.422	0.352
	*113	0.860	0.542	0.783	0.780	0.729	0.560	0.622	0.578	0.648
N		25	36	69	41	48	58	41	32	27
H_e		0.246	0.528	0.343	0.347	0.399	0.497	0.476	0.496	0.465
H_o		0.280	0.361	0.290	0.342	0.375	0.466	0.366	0.531	0.333
*GPI-B**	*85	0.000	0.000	0.007	0.000	0.009	0.000	0.000	0.000	0.000
	*90	1.000	1.000	0.933	1.000	0.990	1.000	1.000	1.000	1.000
N		29	36	75	42	51	59	43	51	29
H_e		0.000	0.000	0.013	0.000	0.020	0.000	0.000	0.000	0.000
H_o		0.000	0.000	0.013	0.000	0.020	0.000	0.000	0.000	0.000
*LDH-B**	*95	0.050	0.030	0.021	0.014	0.020	0.026	0.023	0.000	0.040
	*100	0.950	0.970	0.979	0.986	0.980	0.966	0.977	1.000	0.960
	*112	0.000	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000
N		20	33	72	36	50	58	43	30	25
H_e		0.097	0.060	0.041	0.028	0.040	0.068	0.046	0.000	0.078
H_o		0.000	0.000	0.042	0.028	0.040	0.035	0.047	0.000	0.080
*LDH-C**	*60	0.000	0.000	0.007	0.000	0.000	0.000	0.000	0.000	0.000
	*70	0.000	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000
	*100	1.000	1.000	0.993	0.971	1.000	1.000	1.000	1.000	1.000
N		20	33	71	35	49	58	43	15	23
H_e		0.000	0.000	0.014	0.056	0.000	0.000	0.000	0.000	0.000
H_o		0.000	0.000	0.014	0.057	0.000	0.000	0.000	0.000	0.000

Appendix 4.1.2 (continued)

Locus	Allele	Samples								
		Est.	Coast				Offshore			WS
		D3	Gc	Ze	Ki10	Os	Bli	Nh	Oh	FF
*MDH-B**	**90*	0.000	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000
	**95*	0.000	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000
	**100*	1.000	1.000	1.000	1.000	1.000	0.984	1.000	1.000	1.000
N		31	36	70	39	52	60	43	33	30
H_e		0.000	0.000	0.000	0.000	0.000	0.033	0.000	0.000	0.000
H_o		0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000
*PGM-1**	**70*	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000
	**86*	0.100	0.014	0.054	0.069	0.020	0.073	0.023	0.058	0.033
	**100*	0.880	0.972	0.939	0.903	0.971	0.906	0.965	0.942	0.950
	**114*	0.020	0.014	0.007	0.028	0.009	0.021	0.000	0.000	0.017
N		25	36	74	36	51	48	43	26	30
He		0.212	0.055	0.116	0.182	0.058	0.175	0.069	0.111	0.098
Ho		0.160	0.056	0.122	0.194	0.059	0.188	0.069	0.115	0.100
PGM-2*	**90*	0.025	0.042	0.000	0.023	0.000	0.000	0.018	0.000	0.000
	**103*	0.950	0.944	1.000	0.977	1.000	1.000	0.982	1.000	1.000
	**106*	0.025	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.000
N		20	36	4	22	28	4	27	23	5
He		0.099	0.108	0.000	0.046	0.000	0.000	0.037	0.000	0.000
Ho		0.100	0.056	0.000	0.046	0.000	0.000	0.037	0.000	0.000

Loci *LDH-A**, *MDH-A**, *AK**, *IDHP** and *FH** are fixed for the same allele **100* (cfr. *P. minutus*).

PAPER V

Evidence for fine scale genetic structure and estuarine colonisation in a high gene flow marine goby (*Pomatoschistus minutus*)

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ABSTRACT

Marine fish seem to experience forces where homogenisation of populations should be the rule; yet evidence for structure, even on a small scale, is growing. We assessed genetic diversity and differentiation in 15 samples of the sand goby *Pomatoschistus minutus*, (Pallas 1770) (Gobiidae, Teleostei) from four major environments within the Southern Bight of the North Sea, using seven microsatellite and thirteen allozyme loci. Despite the high dispersal potential of the sand goby, microsatellite loci revealed a moderate level of differentiation (overall F_{ST} = 0.026; overall R_{ST} = 0.058). Both an hierarchical analysis of molecular variance and multivariate analysis revealed a significant differentiation ($P < 0.01$) between estuarine, coastal and marine samples with microsatellites but not with allozymes. Comparison among the different estimators of differentiation (F_{ST} and R_{ST}) pointed to possible historical events and contemporary habitat fragmentation. We conclude that this genetic structure was mainly due to the colonisation of the estuary during its establishment in the early Holocene, and to restricted actual gene flow. All samples can be assigned to two breeding units localised in estuarine and coastal region. Despite the distinction, there were indications of a complex and dynamic spatio-temporal structure, which is most likely, determined by the local current regime and the behavioural ecology of sand gobies.

Keywords: Allele shift; allozymes; gene flow; microsatellites; North Atlantic Ocean; sand goby.

INTRODUCTION

One of the most interesting challenges in marine evolutionary biology is to assess the processes responsible for genetic differentiation of distantly or closely related populations. Consequently, genetic structure of numerous organisms has been assessed using several kinds of estimators, resulting in unequal values for different markers (Pogson et al, 1995. Lemaire et al, 2000; but see Allendorf & Seeb, 2000). Although allozymes are still widely used, microsatellites gained importance due to their highly polymorphic state, facilitating the discovery of subtle differentiation (Shaw et al, 1999; Ruzzante et al, 1998). In addition, they are useful tools to infer more historical determined dispersal and gene flow events, due to molecular insights in the nature of alleles and their mutation models (Balloux & Lugon-Moulin, 2002).

Populations of marine fishes were thought to be genetically homogeneous, e.g. panmictic, for mainly two reasons. First, they usually exhibit a high effective population size and produce a large number of eggs and larvae that disperse through passive or active mechanisms over vast distances, thus limiting population divergence (Wirth & Bernatchez, 2001; Hoarau et al, 2002). Second, the marine environment tends to be physically less structured than the continental systems and to exhibit less constraint for gene flow, rendering marine fishes poor candidates for genetic studies on small geographical scale. Nevertheless, fronts, local and global oceanic current patterns, bottom topography, estuarine influence and climatic barriers restrict the dispersal of pelagic larvae and adults, and promote genetic differentiation within populations (Sinclair, 1988; Bowen & Grant, 1997; Lessios et al, 1999).

Additionally, because of dispersal, many marine species occupy a wide variety of physical environments (Warner, 1997). Such species inhabiting as well marine, as coastal and estuarine regions, are thought to develop a mechanism of "divergence-with-gene-flow" through local adaptation (Beheregaray & Sunnucks, 2001). To test such hypotheses, a system including various types of habitats was chosen to see whether an annual non-commercial small marine fish, with a high reproductive effort and dispersal capability to reach all habitats, is able to develop any reproductive barrier and maintain it.

The sand goby *Pomatoschistus minutus* (Pallas 1770) (Gobiidae, Teleostei), a small bottom-dwelling fish, is well suited to test those hypotheses. It occurs in several European regions and specially within the Southern Bight of the North Sea where it lives in estuarine (Oosterschelde and Westerschelde), coastal and marine habitats where it reproduces from May to July (Fonds, 1973).

Males build a nest and attract females to obtain eggs (Lindström, 1992); they defend the nest until the hatching of the larvae. The larvae are pelagic for 4 to 6 weeks and adopt a demersal life-style after metamorphosis. Adults are thought to have poor swimming abilities, yet they carry out a spawning migration (Pampoulie *et al*, 1999). Given its high dispersal capabilities, we might expect only slight or no genetic differentiation at all among populations living on a small scale. On the other hand, the geomorphology of the Belgian Continental Shelf, characterised by a combination of sand banks and gullies swept by strong tidal currents (De Moor & Lanckneus, 1990), and by an inshore/offshore gradient under the influence of the Schelde estuary (Nihoul & Hecq, 1984; Offringa *et al*, 1996; Dewicke, 2001), might limit dispersal and promote small-scale inter-population differentiation.

The scope of this study is to assess: 1) whether a small marine fish species, exhibiting a high dispersal rate and living in diverse and dynamic environments, could have developed any reproductive barriers in such a heterogeneous area, and 2) whether as suggested by Beheregaray & Sunnucks (2001), those specific hydrodynamic systems lead to a "divergence-with-gene-flow" system which might be favourable to incipient speciation.

MATERIALS AND METHODS

Sampling

Sampling on the Belgian Continental Shelf was carried out with the oceanographic research vessels R/V 'Belgica' and R/V 'Zeeleeuw' along an inshore/offshore gradient in the Coastal area (coastal and Flemish banks: Sb, Ht, and K), the Estuary (Westerschelde), and the marine area (Of1, Of2) over a distance of at most 120 km (Fig. 4.2.1). In addition, four samples were taken in the Schelde estuary (Oosterschelde, The Netherlands) for microsatellite analysis only. The latter area consists of a unique marine environment and is used as a nursery for fish from the adjacent North Sea. Although partly separated from the sea by a dike system, about 80% of the inflow passes through, thus conserving the exchanges between the ecosystems (Hamerlynck & Hostens, 1994). One distant population has been sampled in Texel (Tx; The Netherlands) as outgroup.

Fishes were either frozen in dry ice or liquid nitrogen immediately after capture and kept in

a –80°C freezer until analysis. Gobies were identified morphologically on the basis of the dermal papillae of the head according to Miller (1986), and biochemically according to Wallis and Beardmore (1984a,b).

Table 4.2.1. Sampling locations of *Pomatoschistus minutus* in the Southern Bight of the North Sea.

Habitat	Sampling site	*Code*	Period	*Allozymes*	*Microsatellites*
Westerschelde	Doel 10	W1	October 1998	71	54
	Westerschelde	W2	August 2001	-	52
Oosterschelde	Oosterschelde 7	Oc7	August 2001	-	52
	Oosterschelde 15	Oc15	August 2001	-	52
	Oosterschelde 21	Oc21	August 2001	-	45
	Oosterschelde 37	Oc37	August 2001	-	52
Coast	Kwintebank 2	K2	February 1997	68	-
	Stroombank	Sb	February 1997	83	-
	Kwintebank 10	K1	October 1997	161	58
	Raan 1	Ra1	March 1997	50	-
	Ostend	Os	October 1997	52	-
	Weststroombank	Ht	October 1998	35	-
	Kwintebank	K12	August 2000	-	53
	Oostduinkerke	Ot1	August 2000	-	54
	Zuid-Steenbank	Zs	August 2000	-	53
	Raan 2	Ra2	August 2000	-	53
	Niewpoort	Np	August 2000	-	52
	Oostduinkerke	Ot2	August 2001	-	52
	Texel	Tx	September 1999	-	51
Marine	Oosthinder	Of1	October 1997	51	36
	Bligh Bank	Of2	February 1998	28	-

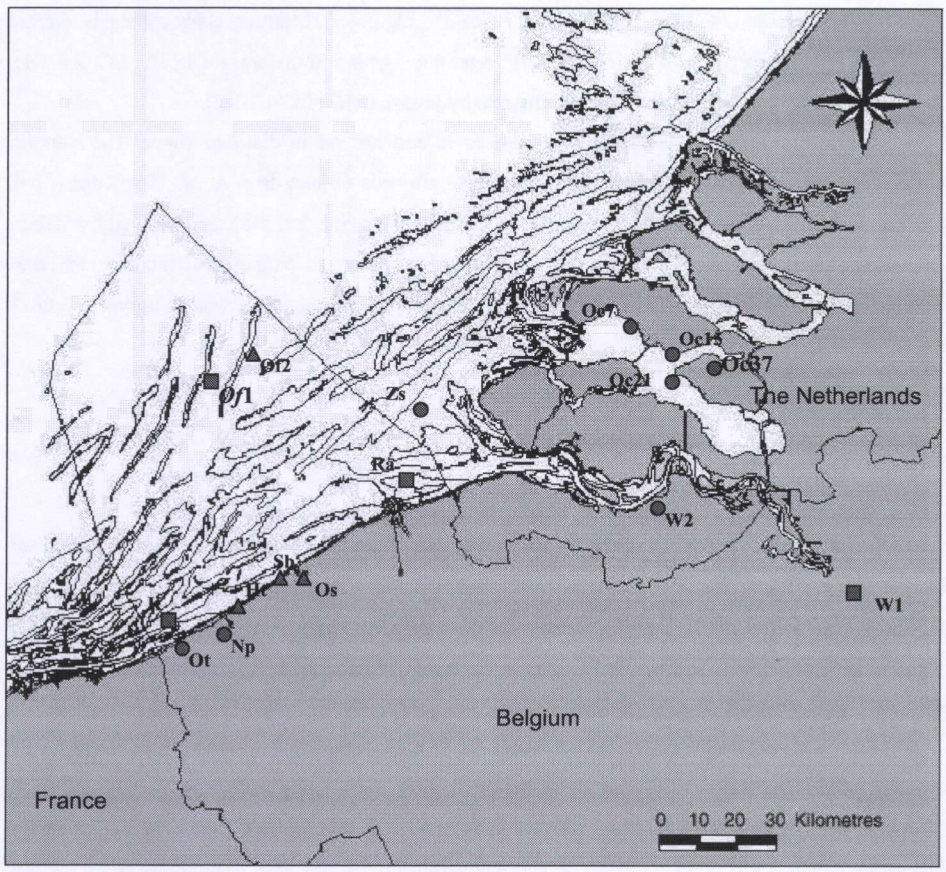


Figure 4.2.1. Sampling locations and codes of the sampling sites of *Pomatoschistus minutus* within the Southern Bight of the North Sea. Triangles: allozyme samples; Squares: microsatellite and allozyme loci; Circles: microsatellite samples. For the code designation, see Table 4.2.1.

Allozyme genotyping

Allelic variation was assayed for 9 populations at 8 enzymes coding for 13 loci, namely Creatine kinase (*CK-A**, EC 2.7.3.2), Lactate dehydrogenase (*LDH-A**, EC 1.1.1.27; *LDH-B**, EC 1.1.1.27;

*LDH-C**, EC 1.1.1.27), Malate dehydrogenase (*MDH-A**, EC 1.1.1.37; *MDH-B**, EC 1.1.1.37), Phosphoglucomutase (*PGM-1**, EC 5.4.2.2; *PGM-2**, EC 5.4.2.2), Glucose phosphate isomerase (*GPI-A**, EC 5.3.1.9; *GPI-B**, EC 5.3.1.9), Aspartate amino transferase (*AAT**, EC 2.6.1.1), Adenylate kinase (*AK**, EC 2.7.4.3) and Fumarate hydratase (*FH**, EC 4.2.1.2).

Liver, eye and muscle tissues were dissected and ground in distilled water. The samples were subjected to cellulose acetate allozyme electrophoresis (Richardson *et al*, 1986) using two continuous buffer systems: Tris-maleate (pH 7.8) and Tris-glycine (pH 8.8) as described by Hebert & Beaton (1989) with modification. Loci were stained according to recipes described by Hebert & Beaton (1989) and Richardson *et al* (1986). The fastest migrating locus was designated 1 or A according to the nomenclature of Shaklee *et al* (1990).

Microsatellite genotyping

Allelic variation was assayed at seven microsatellite loci, *Pmin-01*, *Pmin-05* and *Pmin-10* described by Jones *et al* (2001a, b), and the newly developed loci *Pmin-06*, *Pmin-07*, *Pmin-08* and *Pmin-11* (Table 4.2.2). A total of 15 samples were assayed with a sampling size of 36-54 individuals per population (Table 4.2.1). DNA samples were extracted from fin clips using a Chelex (Biorad, 10%) extraction protocol (Walsh *et al*, 1991).

For all primer sets used, PCR was conducted in a 10 µl reaction volumes containing specific amounts of primers and $MgCl_2$ ranging respectively from 1 to 2 µM and 0.6 to 2 mM. All PCR reactions were preceded by an initial denaturation step of 2 min at 95°C followed by 25 cycles extension steps: 1 min at 95°C, 1 min at the annealing temperature (60°C for *Pmin-01*; 62°C for *Pmin-05* and *Pmin-10*, 54°C for *Pmin-06*, 57°C for *Pmin-07*, 56°C for *Pmin-08* and 60°C for *Pmin-11*) and 1 min at 72°C. A final elongation step of 3 min at 72°C was performed.

PCR products were diluted with 5 µl (1:3) of stop-loading solution (formamide 99% and bromophenol blue) and were electrophoresed on 25 cm 6% polyacrylamide gels and detected on an automatic sequencer (LI-COR, model 4200) using the software E-seq ver. 2.00 (LI-COR Inc., 2001). Products were scored using the software Gene ImagIR ver. 4.03 (Scanalytics Inc., 2001) several times to avoid scoring errors. Suspect individuals were deleted from the analysis.

Table 4.2.2. Characteristics of the microsatellite DNA markers scored on 15 populations of sand goby (768 individuals were scored). AN: Accesion Number.

Locus	Primer sequence (5'→3')	Repeat Sequence	Size range	No alleles	AN
Pmin-01	R: CACAAAGTCAATCCTAAATA	$(GT)_{41}$	158-418	86	AF516896
	F: CCAAACTGTTTAGCACTG				
Pmin-05	R: TTTCCCCCGAACAACACAAC	$(GT)_{31}$	118-276	88	AF516897
	F: TTCCCATGCCTCCTTTTGTC				
Pmin-06	R: CGCATTAGAATTATTAGGCC	$(CA)_{14}(AA)(CA)_{20}$	91-199	43	AF516898
	F: TCANTNCTACTCACTAACCT				
Pmin-07	R: TTTCAGCTGTATAGTCGCTGC	$(CA)_{10}$	162-178	9	AF516899
	F: TCGACAAACTCAAACTCACC				
Pmin-08	R: GTTCGCCACCATGCACC	$(CA)_9(CG)(CA)_2(C$	152-286	45	AF516900
	F: AGTCTTCCACCGCTCACG	$G)(CA)_2$			
Pmin-10	R: AACCGCCCAATCCACAAC	$(GT)_{25}$	142-202	45	AF516901
	F: GAATGTCCCGAGAAACTGGAG				
Pmin-11	R: CCGACCCAGAAATGGACAA	$(TGGA)_6$	100-120	8	AF516902
	F: GATTCGCCAACACAGATTCAA				

Genetic data analysis

Allele frequencies, observed (H_O) and unbiased expected heterozygosity (H_e) were calculated in GENETIX version 4.02 (Belkhir et al, 2001). Hardy-Weinberg equilibrium (HWE) was calculated and tested for significance in GENEPOP version 3.1 (Raymond & Rousset, 1995). Significance levels were adjusted with a sequential Bonferroni test (Rice, 1989). Wright's single-locus F-statistics (Wright, 1969) were calculated from allele frequencies for all loci examined for each population according to Weir & Cockerham (1984) in GENETIX (θ). For the microsatellite loci, differentiation between populations was also quantified using the analogue rho of the R_{ST} of Slatkin (1995) following Goodman (1997) using the computer program RSTCALC (Goodman, 1997) and assuming the Stepwise Mutation Model (SMM; Kimura & Otha, 1978). Standard deviations of single-locus F_{ST} values were obtained by jack-knifing over all populations according to Weir (1990). Significance of multi-locus F_{ST} and R_{ST} was assessed with permutation tests (1000 replicates). Pairwise genetic distances corrected for bias in sampling (Nei, 1978) was calculated in GENETIX

assuming genetic drift-mutation equilibrium and a constant population size over time for both allozyme and microsatellite loci. For microsatellite loci, genetic distance between populations was also assessed by the specific distance $\partial\mu^2$ of Goldstein *et al* (1995) obtained with RSTCALC. Genetic linkage disequilibrium between locus pairs was estimated according to Weir and Cockerham (1984) and tested on contingency tables under the null hypothesis of independence. A microsatellite multilocus estimate of the effective number of migrants (Nm) according to Slatkin's private allele model (1985) was assessed with GENEPOP. We performed a Mantel test (Mantel, 1967) to test for correlation between geographical and genetic distance between samples (isolation-by-distance) as implemented in GENETIX (after 1000 permutations). An Analysis of Molecular Variance (AMOVA) was carried out in ARLEQUIN version 2.0 (Schneider *et al*, 2000) to assess hierarchical partitioning of genetic variability within and among populations, and among post-hoc defined regions (Oosterschelde, Westerschelde, coastal and marine). We carried out a multidimensional scaling (MDS) approach on pairwise genetic distance (Nei, 1978; Goldstein et al, 1995) using Statistica 5.1 (Statsoft Inc, 1997).

RESULTS

Allozyme genetic diversity

Nine out of 13 scored allozyme loci were polymorphic in the nine samples analysed (*LDH-A*, LDH-B*, LDH-C*, MDH-1*, PGM-1*, PGM-2*, GPI-A*, GPI-B** and *AAT**; Table 4.2.3). The observed heterozygosity averaged over all loci ranged from 0.09 to 0.11. No inter-population differences in mean heterozygosity, number of alleles per locus or levels of polymorphism were observed (Table 4.2.3).

Table 4.2.3. Number of individuals scored (n), number of alleles (A), mean number of alleles (MNA), expected heterozygosity (H_E), observed heterozygosity (H_O) and F_{IS} according to Weir & Cockerham (1984) for the polymorphic allozyme loci of sand goby. Bold values: F_{IS} values deviating significantly from HWE after sequential Bonferroni corrections. For sampling codes, see Table 4.2.1.

Locus	**Samples**								
	K1	**K2**	**Os**	**Ra1**	**Sb**	**Ht**	**Of1**	**Of2**	**W1**
LDH-A*									
n	155	60	51	50	79	35	44	28	57
A	2	2	2	1	1	1	2	1	2
H_E	0.013	0.017	0.019	0.000	0.000	0.000	0.023	0.000	0.017
H_O	0.013	0.017	0.020	0.000	0.000	0.000	0.023	0.000	0.017
F_{IS}	-0.003	0	0	-	-	-	0	-	0
LDH-B*									
n	146	56	51	49	77	35	36	28	66
A	1	2	2	2	1	1	1	2	1
H_E	0.000	0.018	0.038	0.020	0.000	0.000	0.000	0.069	0.000
H_O	0.000	0.018	0.039	0.020	0.000	0.000	0.000	0.070	0.000
F_{IS}	-	0	0	0	-	-	-	-0.019	-
LDH-C*									
n	144	54	51	47	66	35	19	28	64
A	4	3	3	3	3	3	3	3	3
H_E	0.549	0.561	0.573	0.517	0.481	0.231	0.460	0.428	0.424
H_O	0.403	0.500	0.373	0.298	0.439	0.171	0.105	0.214	0.281
F_{IS}	**0.150**	**0.307**	**0.288**	**0.284**	**0.298**	**0.311**	**0.502**	**0.513**	**0.239**
MDH-1*									
n	156	57	52	49	56	35	43	28	71
A	2	1	2	1	2	1	3	1	1
H_E	0.013	0.000	0.019	0.000	0.018	0.000	0.068	0.000	0.000
H_O	0.013	0.000	0.019	0.000	0.018	0.000	0.070	0.000	0.000
F_{IS}	-0.003	-	0	-	0	-	-0.006	-	-

Locus	Samples								
	K1	K2	Os	Ra1	Sb	Ht	Of1	Of2	W1
PGM-1*									
n	153	56	49	49	61	31	40	24	65
A	3	3	3	3	2	3	3	3	3
H_E	0.284	0.180	0.250	0.265	0.290	0.297	0.258	0.284	0.242
H_O	0.307	0.161	0.184	0.245	0.246	0.290	0.300	0.333	0.215
F_{IS}	-0.045	0.076	0.131	0.021	0.147	0.029	-0.077	-0.154	0.071
PGM-2*									
N	105	47	29	38	44	25	38	9	51
A	5	3	4	4	3	3	4	3	6
H_E	0.595	0.567	0.620	0.606	0.600	0.547	0.630	0.512	0.571
H_O	0.476	0.532	0.552	0.658	0.728	0.360	0.579	0.222	0.549
F_{IS}	0.204	0.072	0.128	-0.073	-0.201	0.360	0.095	0.605	0.049
GPI-A*									
n	156	61	51	50	66	34	46	24	70
A	3	1	1	1	1	2	2	1	2
H_E	0.013	0.000	0.000	0.000	0.000	0.057	0.022	0.000	0.042
H_O	0.013	0.000	0.000	0.000	0.000	0.059	0.022	0.000	0.043
F_{IS}	0	-	-	-	-	-0.016	0	-	-0.015
GPI-B*									
n	157	61	51	50	72	34	45	24	70
A	2	2	2	3	2	1	2	1	2
H_E	0.056	0.016	0.019	0.078	0.027	0.000	0.022	0.000	0.056
H_O	0.057	0.016	0.020	0.080	0.028	0.000	0.022	0.000	0.043
F_{IS}	-0.026	0	0	-0.011	-0.007	-	0	-	0
GOT*									
n	114	45	47	28	64	35	44	26	71
A	1	1	1	2	1	2	1	1	2
H_E	0.000	0.000	0.000	0.035	0.000	0.028	0.000	0.000	0.014
H_O	0.000	0.000	0.000	0.036	0.000	0.029	0.000	0.000	0.014
F_{IS}	-	-	-	0	-	0	-	-	0
Multi-locus									
MNA	2.15	1.69	1.85	1.77	1.54	1.62	1.92	1.58	2.08
H_E	0.118	0.104	0.118	0.117	0.109	0.089	0.114	0.099	0.105
H_O	0.099	0.096	0.093	0.103	0.112	0.070	0.086	0.065	0.090

All polymorphic loci were in HW equilibrium after Bonferroni correction with the exception of *LDH-C**, where a strong heterozygote deficit across all samples was observed, independent of sample size (ranging from 30 to 200 individuals). Neither a trend nor gradient in allele frequencies across sampling sites was observed at this locus. A Mantel test failed to show any correlation between Nei's (1978) genetic distances and geographic distances ($P > 0.05$ under null hypothesis after 1000 permutations).

Allozyme population structure

As no clear differentiation was observed between all samples separately, samples were grouped by season (summer and winter) to assess temporal variation and variation of the *F*-estimates. The multi-locus F_{ST} value (0.01) for 'summer-autumn' samples (Of1, Os, K1, W1 and Ra1) was significant ($P < 0.05$), which was entirely due to a differentiation at locus *LDH-C** ($F_{ST} = 0.028$, $P < 0.05$). Excluding *LDH-C**, the multi-locus F_{ST} was only 0.003 (not significant). Exact tests confirmed the differentiation at *LDH-C** ($P =$ "0.0001). The 'winter' samples (Ra3, Of2, Sb and K2) were less differentiated ($F_{ST} = 0.005$) than the 'summer' samples (F_{ST} not significant). No differentiation at locus *LDH-C** was observed in this group.

Pair-wise genetic distances (Nei, 1978) calculated over all loci between the samples of sand goby were not significant. Temporal variation in allele frequencies was assessed by comparing samples taken at approximately the same site in two different seasons. K2 was compared with K1, Sb with Ht and Oh with Of2 (Fig. 4.2.1). Exact tests for allelic homogeneity (Raymond & Rousset, 1995) showed no differences. The AMOVA revealed that within-population effects explained all the observed variation and did not show any consistent differentiation between the samples (Table 4.2.4). The MDS did reveal a slight differentiation between marine, estuarine (Westerschelde) and coastal samples (Figure 4.2.2a).

Table 4.2.4. Hierarchical analysis of molecular variance (AMOVA) among 9 populations of *Pomatoschistus minutus* grouped in three regional groups for the allozyme data (Marine, Coastal and Westerschelde) and 15 populations grouped in four regional groups for the microsatellite data (Marine, Coastal, Westerschelde and Oosterschelde). df: degree of freedom, *p*: significance level.

Loci	Source of variation	df	Variance components	% variation	Fixation indices	P
Allozymes	Among groups	2	-0.003	-0.83	CT=-0.008	0.536
	Among samples within groups	6	0.035	8.93	SC=0.089	<0.0001
	Within samples	899	0.356	91.90	ST=0.081	<0.0001
	Total	907	0.387	100		
Microsatellites	Among groups	3	0.017	0.60	CT=0.006	<0.01
	Among samples within groups	11	0.066	2.28	SC=0.022	<0.0001
	Within samples	1519	2.79	97.12	ST=0.029	<0.0001
	Total	1533	2.88	100		

Microsatellite genetic diversity

Although the seven microsatellite loci studied exhibited a high level of polymorphism (Table 4.2.2; $P_{(0.95)} = 1$), two of them might be considered as highly polymorphic (*Pmin-01* and *Pmin-05*) while three loci are moderately polymorphic (*Pmin-06*, *Pmin-08* and *Pmin-10*) and two slightly polymorphic (*Pmin-07* and *Pmin-11*) compared to values found in the literature. The number of alleles per locus across all samples ranged from 8 (*Pmin-11*) to 88 (*Pmin-05*). Observed heterozygosity averaged over all loci ranged from 0.62 to 0.76 in the 15 samples and tended to be lower than the expected heterozygosity. Genotypic proportions in 55 of 105 exact tests were out of HWE (Table 4.2.5). In particular, at *Pmin-10* no HWE was observed in any sample excepted for K1. The overall excess of homozygotes for all loci combined (F_{IS}), as quantified by the correlation of alleles within individuals was 0.163 (Table 4.2.6). Based on permutation tests (1000 replicates), the F_{IS} values were significant for six out of seven loci ($0.001 < P < 0.01$).

Table 4.2.5. Number of individuals scored (n), number of alleles (A), mean number of alleles (MNA), expected heterozygosity (H_E), observed heterozygosity (H_O) and F_{IS} according to Weir & Cockerham (1984) for the microsatellite loci. Bold values: F_{IS} values deviating significantly from HWE after sequential Bonferroni corrections. For sampling codes, see Table 4.2.1.

	Samples														
Locus	**K1**	**K12**	**Zs**	**Ra2**	**Ot1**	**Ot2**	**Np**	**Tx**	**Of1**	**W1**	**W2**	**Oc7**	**Oc15**	**Oc21**	**Oc37**
Pmin-01															
n	56	53	49	52	53	48	49	49	36	52	47	50	50	41	52
A	45	33	34	37	49	39	43	46	18	49	46	46	38	25	46
H_E	0.967	0.939	0.953	0.954	0.971	0.958	0.965	0.967	0.940	0.968	0.963	0.966	0.962	0.936	0.970
H_O	0.696	0.623	0.674	0.750	0.868	0.834	0898	0.776	0.833	0.808	0.787	0.860	0.960	0.927	0.904
F_{IS}	0.288	**0.345**	**0.303**	0.223	0.116	0.141	0.08	**0.208**	0.105	0.175	**0.193**	0.120	**0.012**	0.022	0.079
Pmin-05															
n	57	50	52	53	54	51	50	51	36	54	51	49	49	43	52
A	56	37	32	39	44	33	40	39	22	46	38	31	40	39	26
H_E	0.976	0.956	0.947	0.962	0.966	0.959	0.962	0.964	0.940	0.967	0.964	0.950	0.964	0.961	0.942
H_O	0.860	0.660	0.635	0.755	0.926	0.843	0.860	0.765	0.889	0.815	0.843	0.714	0.755	0.977	0.689
F_{IS}	0.128	**0.319**	**0.338**	**0.224**	0.051	**0.131**	**0.116**	**0.216**	**0.069**	**0.166**	**0.135**	**0.258**	**0.227**	-0.004	**0.279**
Pmin-06															
n	57	52	53	53	54	51	51	49	36	53	52	52	48	44	49
A	29	27	23	25	22	26	20	24	19	27	24	13	13	24	30
H_E	0.911	0.914	0.919	0.925	0.909	0.930	0.904	0.920	0.882	0.886	0.871	0.723	0.833	0.902	0.863
H_O	0.860	0.679	0.736	0774	0.722	0.846	0.843	0.612	0.750	0.887	0.904	0.750	0.750	0.818	0.694
F_{IS}	0.065	**0.266**	**0.208**	**0.173**	**0.214**	**0.100**	**0.077**	**0.344**	0.164	0.009	-0.028	-0.028	**0.109**	**0.104**	**0.205**
Pmin-07															
n	57	53	52	53	54	50	47	46	36	53	50	52	51	43	52
A	6	6	6	6	5	5	5	7	3	7	5	6	6	6	6
H_E	0.697	0.702	0.607	0.700	0.652	0.634	0.607	0.764	0.538	0.704	0.607	0.691	0.641	0.499	0.606
H_O	0.386	0.660	0.442	0.697	0.611	0.680	0.500	0.717	0.679	0.453	0.500	0.500	0.451	0.465	0.673
F_{IS}	**0.453**	**0.069**	0.280	0.039	**0.072**	-0.069	**0.207**	0.072	0.239	**0.365**	0.186	**0.286**	**0.305**	0.079	-0.102
Pmin-08															
n	56	52	53	49	52	51	46	47	36	54	50	48	48	45	52
A	29	28	25	25	22	26	26	25	20	23	24	21	30	27	29
H_E	0.936	0.894	0.900	0.924	0.879	0.913	0.914	0.928	0.888	0.926	0.918	0.923	0.946	0.930	0.940
H_O	0.857	0.884	0.849	0.816	0.692	0.922	0.804	0.872	0.889	0.833	0.900	0.771	0.792	0.910	0.810
F_{IS}	**0.094**	0.020	0.064	0127	**0.221**	0.001	0.131	0.070	0.013	0.110	0.030	**0.175**	**0.174**	**0.031**	**0.149**
Pmin-10															
n	58	53	53	53	54	52	50	51	36	53	50	52	50	45	52
A	28	32	14	26	24	26	22	21	19	28	28	18	22	30	24
H_E	0.931	0.931	0.853	0.929	0.931	0.947	0.941	0.905	0.879	0.941	0.939	0.909	0.903	0.929	0.931
H_O	0.828	0.660	0.556	0.577	0.860	0.706	0.726	0.580	0.667	0.648	0.686	0.689	0.680	0.591	0.583
F_{IS}	0.120	**0.368**	**0.345**	**0.387**	**0.086**	**0.299**	**0.278**	**0.368**	**0.255**	**0.319**	**0.278**	**0.100**	**0.257**	**0.374**	**0.382**
Pmin-11															
n	58	53	53	53	54	52	50	51	36	53	50	52	50	45	52
A	7	5	6	7	6	7	7	6	5	6	5	5	6	7	6
H_E	0.596	0.543	0.518	0.552	0.598	0.587	0.598	0.585	0.157	0.513	0.439	0.623	0.550	0.533	0.470
H_O	0.552	0.566	0.547	0.547	0.648	0.592	0.700	0.412	0.167	0.418	0.460	0.712	0.600	0.511	0.500
F_{IS}	0.083	0.305	-0047	-0.051	-0.074	-0.090	-0.160	**0.305**	-0.045	0.089	-0.039	-0.132	-0.081	0.052	-0.057
Total															
MNA	28.57	24.00	20.00	23.57	24.57	23.00	24.29	24.00	15.14	26.57	24.29	20.00	22.14	22.57	22.43
H_E	0.859	0.840	0.813	0.849	0.844	0.846	0.834	0.862	0.743	0.843	0.814	0.827	0.828	0.813	0.817
H_O	0.720	0.676	0.636	0.705	0.761	0.764	0.726	0.676	0.659	0.702	0.726	0.615	0.713	0.743	0.693

Exact tests for linkage-disequilibrium yielded several significant values ($0.01 < P < 0.05$) involving several pairs of loci without any consistency, thus suggesting that the results were not due to physical linkage of the marker loci. No linkage disequilibrium was observed between allozyme and microsatellite loci in the three common sampling sites (Of1, K1 and W1).

Table 4.2.6. *F*-statistics (GENETIX) and *R*-statistics (RSTCALC) over loci for seven microsatellite loci in 15 populations of *Pomatoschistus minutus*.

	***F*-statistics**			***R*-statistics**		
Locus	F_{IS}	F_{IT}	F_{ST}	R_{IS}	R_{IT}	R_{ST}
Pmin-01	0.165	0.174	0.011	**0.192**	**0.226**	**0.044**
Pmin-05	0.179	0.191	0.015	0.191	0.227	0.050
Pmin-06	0.134	0.174	0.046	0.078	0.167	0.106
Pmin-07	0.170	0.221	0.061	0.109	0.240	0.148
Pmin-08	0.096	0.102	0.007	0.108	0.120	0.015
Pmin-10	0.330	0.351	0.031	0.451	0.462	0.021
Pmin-11	-0.007	0.011	0.012	0.072	0.086	0.016
Total	0.163	0.185	0.026	0.197	0.233	0.058
CI 95%	0.151-0.172	0.172-0.194	0.024-0.028			

Microsatellite population structure

The partitioning of genetic variance among and within the 15 populations as estimated by *F*-statistics showed a mean F_{ST} value of 0.026 and a F_{IS} of 0.163 while *R*-statistics showed a mean R_{ST} value of 0.058 and a R_{IS} of 0.197 (Table 4.2.6). Pair-wise differentiation between populations yielded significant F_{ST} values for all comparisons after sequential Bonferroni adjustment, while not all values were significant for the R_{ST} estimator (Table 4.2.6). The highest pair-wise F_{ST} and R_{ST} values were observed between the marine populations and the other populations. The total differentiation for both estimators was mainly due to loci *Pmin-06* and *Pmin-07* (Table 4.2.6). Nei's (1978) and $\partial\mu^2$ (Goldstein *et al*, 1995) distances exhibited significant values between all pairs of populations after Bonferroni correction (Table 4.2.7). The highest values were observed between marine populations and the others, and to a lesser extent between the estuary (Oosterschelde) and coastal populations.

Table 4.2.7. Estimates of F_{ST} and R_{ST} among pairs of populations of *Pomatoschistus minutus*. F_{ST} estimates for 7 microsatellite loci are above the diagonal and R_{ST} are below the diagonal. All F_{ST} values remained significant after Bonferroni adjustement; * indicated significant values of R_{ST}.

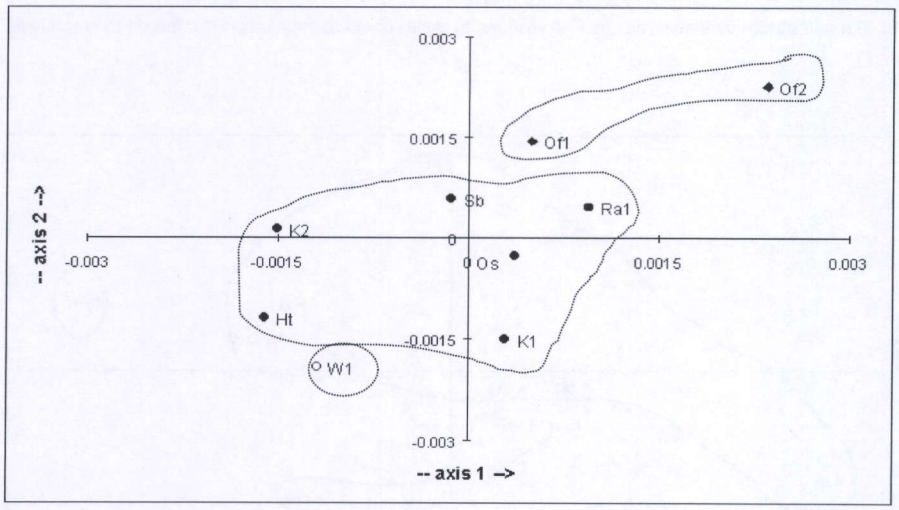
	K1	K12	Zs	Ra2	Ot1	Ot2	Np	Tx	Of1	W1	W2	Oc7	Oc15	Oc21	Oc37
K1	0	0.017	0.025	0.014	0.014	0.018	0.035	0.017	0.049	0.010	0.021	0.032	0.020	0.022	0.022
K12	0.085*	0	0.025	0.014	0.014	0.013	0.036	0.014	0.051	0.015	0.019	0.032	0.021	0.026	0.021
Zs	0.060*	0.013	0	0.017	0.022	0.017	0.035	0.036	0.060	0.021	0.023	0.040	0.025	0.018	0.025
Ra2	0.048*	0.030*	0.013	0	0.008	0.017	0.033	0.016	0.034	0.014	0.018	0.023	0.014	0.027	0.019
Ot1	0.034*	0.047*	0.025*	0.000	0	0.027	0.040	0.013	0.037	0.015	0.025	0.032	0.020	0.035	0.028
Ot2	0.082*	-0.003	0.023*	0.040*	0.063*	0	0.018	0.019	0.064	0.015	0.018	0.024	0.019	0.014	0.016
Np	0.107*	0.049*	0.100*	0.103*	0.099*	0.043*	0	0.029	0.085	0.031	0.031	0.036	0.037	0.021	0.025
Tx	0.074*	0.014	0.041*	0.041*	0.049*	0.008	0.020	0	0.042	0.019	0.023	0.030	0.023	0.039	0.025
Of1	0.099*	0.071*	0.038*	0.027*	0.043*	0.092*	0.148*	0.097*	0	0.042	0.057	0.059	0.051	0.073	0.055
W1	0.022*	0.032*	0.023*	0.010	0.008	0.037*	0.082*	0.053*	0.044*	0	0.022	0.017	0.011	0.019	0.014
W2	0.053*	0.019	0.041*	0.036*	0.048*	0.022*	0.049*	0.027*	0.107*	0.024	0	0.045	0.027	0.025	0.022
Oc7	0.112*	0.056*	0.096*	0.077*	0.084*	0.080*	0.100*	0.100*	0.126*	0.055*	0.065*	0	0.018	0.034	0.026
Oc15	0.085*	0.042*	0.076*	0.068*	0.080*	0.056*	0.072*	0.081*	0.124*	0.038*	0.020	0.017	0	0.027	0.016
Oc21	0.033*	0.029*	0.031*	0.032*	0.034*	0.029*	0.074*	0.033*	0.104*	0.009*	0.013	0.078*	0.053*	0	0.014
Oc37	0.081*	0.005	0.037*	0.041*	0.055*	0.008	0.055*	0.019	0.105*	0.031*	0.024	0.044*	0.040*	0.015	0

Table 4.2.8. Estimates of Nei (1978) and $\partial\mu^2$ of Goldstein *et al.* (1995) distances among pairs of populations of *Pomatoschistus minutus*. Nei's distances estimates for 7 microsatellite loci are below the diagonal and $\partial\mu^2$ distances are above the diagonal. All values remained significant after Bonferroni adjustement. For sampling codes, see Table 1.

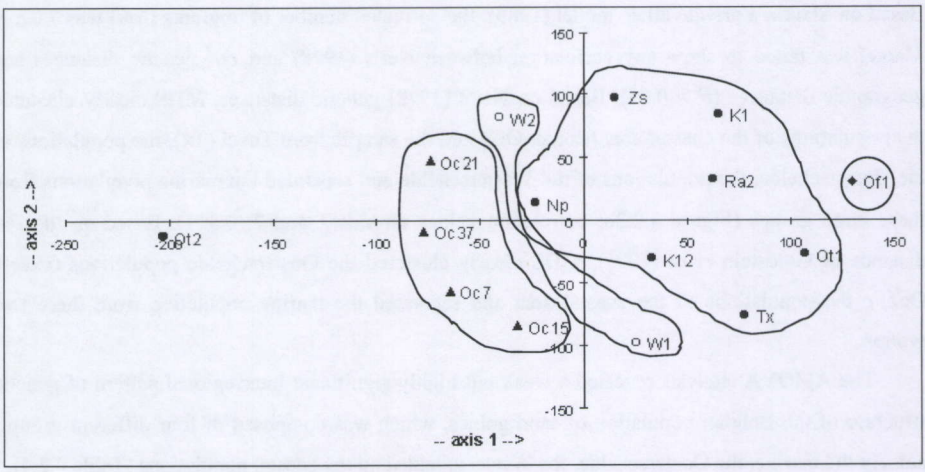
	K1	K12	Zs	Ra2	Ot1	Ot2	Np	Tx	Of1	W1	W2	Oc7	Oc15	Oc21	Oc37
K1	0	0.061	0.041	0.039	0.026	0.060	0.094	0.059	0.063	0.024	0.045	0.090	0.073	0.025	0.058
K12	0.120	0	0.010	0.022	0.028	0.002	0.032	0.013	0.038	0.020	0.016	0.025	0.028	0.020	0.007
Zs	0.148	0.148	0	0.010	0.015	0.014	0.069	0.030	0.019	0.014	0.025	0.042	0.044	0.021	0.021
Ra2	0.101	0.100	0.102	0	0.003	0.025	0.083	0.039	0.015	0.009	0.023	0.034	0.038	0.020	0.025
Ot1	0.100	0.096	0.129	0.058	0	0.031	0.085	0.045	0.019	0.005	0.025	0.034	0.037	0.017	0.027
Ot2	0.132	0.087	0.098	0.120	0.183	0	0.025	0.007	0.046	0.023	0.015	0.031	0.032	0.018	0.007
Np	0.245	0.240	0.205	0.222	0.273	0.117	0	0.016	0.120	0.066	0.032	0.062	0.048	0.046	0.033
Tx	0.139	0.105	0.234	0.121	0.094	0.143	0.205	0	0.072	0.039	0.020	0.053	0.048	0.023	0.017
Of1	0.216	0.233	0.272	0.135	0.146	0.309	0.448	0.178	0	0.025	0.054	0.057	0.063	0.057	0.054
W1	0.073	0.098	0.126	0.099	0.098	0.099	0.204	0.136	0.176	0	0.015	0.025	0.022	0.010	0.018
W2	0.126	0.111	0.126	0.107	0.142	0.100	0.181	0.135	0.251	0.129	0	0.024	0.010	0.011	0.018
Oc7	0.220	0.206	0.239	0.152	0.210	0.155	0.229	0.211	0.274	0.107	0.268	0	0.010	0.032	0.017
Oc15	0.132	0.133	0.145	0.089	0.125	0.078	0.233	0.154	0.224	0.069	0.155	0.110	0	0.027	0.025
Oc21	0.128	0.155	0.099	0.164	0.207	0.119	0.119	0.257	0.342	0.113	0.135	0.200	0.153	0	0.011
Oc37	0.136	0.124	0.140	0.113	0.169	0.093	0.145	0.153	0.245	0.082	0.121	0.150	0.093	0.076	0

Based on Slatkin's private allele model (1985), the corrected number of migrants (*Nm*) was 7.07. A Mantel test failed to show any correlation between Nei's (1978) and $\partial\mu^2$ genetic distances and geographic distances ($P > 0.05$). Based on Nei's (1978) genetic distances, MDS clearly clustered the populations of the coastal area (except Ot2) and the sample from Texel (Tx), the populations of the Oosterschelde, the populations of the Westerschelde and separated the marine populations from these three groups (Figure 4.2.2b; correlation values on axis 1 and 2: 0.311). Based on the $\partial\mu^2$ distance of Goldstein *et al* (1995), MDS clearly clustered the Oosterschelde populations (except Oc21), the populations of the coastal area and separated the marine population from these two groups.

The AMOVA analysis revealed a weak but highly significant interregional pattern of genetic structure of the Belgian population of sand gobies, which was composed of four different groups, namely the marine, the Oosterschelde, the Westerschelde and the coastal populations (Table 4.2.4).



(a)



(b)

Figure 4.2.2. Multidimensional scaling analysis on populations of *Pomatoschistus minutus* based on Nei's distances (1978). a) results observed for allozymes on 9 populations; b) results observed for microsatellite loci on 15 populations.

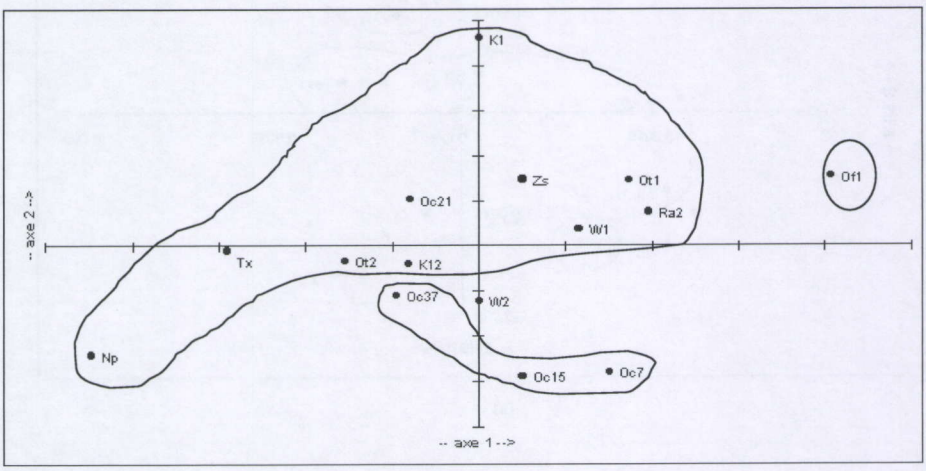


Fig. 4.2.3: Multidimensional scaling analysis on 15 populations of *Pomatoschistus minutus* based on the $\partial\mu^2$ distance of Goldstein *et al* (1995).

DISCUSSION

An unusual genetic diversity and heterozygote deficiency

The degree of heterozygosity as assayed with allozymes was comparable to values compiled for marine fish species (Ward *et al,* 1994) and other gobioid fishes (Geertjes *et al*, 2001). Wallis and Beardmore (1984a) found slightly lower values in sand goby but studied more loci of which a fairly large proportion was monomorphic, thus decreasing overall values of heterozygosity. Polymorphism at most allozyme loci was comparable to the results of Wallis and Beardmore (1984a), with the exception of the highly polymorphic *LDH-C*,* which was completely monomorphic in the former study, probably due to the use of starch gel electrophoresis.

The genetic diversity of the microsatellite loci assessed as the expected level of heterozygosity H_E exhibited a wide range (0.157-0.976) and was generally comparable to other marine fish species (García de León *et al*, 1997; Ruzzante *et al*, 1998; De Innocentiis *et al*, 2001) except for loci *Pmin-07* and *Pmin-11*. Striking is the polymorphism of the loci *Pmin-01* (86 alleles), *Pmin-05* (88 alleles) and *Pmin-10* (45 alleles) being much higher than in any other species studied (DeWoody & Avise, 2000). This generally high polymorphism might be linked to the different environmental conditions along the Belgian Continental Shelf, thus maintaining high genetic polymorphism of its inhabitants. In fact, genetic diversity is regarded as an important asset to cope with the variety of environmental states (Levene 1953).

Most microsatellite loci and the allozymatic *LDH-C** locus clearly show a deficit in heterozygotes. This pattern is not unusual in populations of marine organisms as shown in several studies (Smith, 1987; García de León *et al*, 1997; De Innocentiis *et al*, 2001), and might find its origin in scoring errors, the Wahlund effect (Wahlund, 1928), inbreeding, the occurrence of null alleles, assortative mating or selection against heterozygotes.

The Wahlund effect, the most common explanation of heterozygote deficiency, should result in significant F_{IS} values at more than one allozyme locus, as drift causing population structuring should affect all polymorphic loci equally (Pogson *et al*, 1995). For the microsatellites this hypothesis can be rejected as well, because the level of differentiation observed between samples (F_{ST} = 0.026; R_{ST} = 0.058) was by far smaller than the mean F_{IS} value (F_{IS} = 0.163; $R_{I\,S}$= 0.197) (García de León *et al*, 1997). Moreover, when populations were grouped following the AMOVA defined structure, the overall F_{IS} value did not decrease, excluding the Wahlund effect as

explanation.

Inbreeding remains an unlikely explanation in fish with large populations such as gobies which are not subject to drastic reduction in their effective population sizes like commercial fishes (Fu *et al*, 2001; Hoarau *et al*, 2002). Nevertheless, polygyny and polyandry, which are known to be common features in gobies (Lindström & Seppä, 1996), could be responsible for inbreeding because some of the parents might dominate the progeny (see Zeh & Zeh, 2001). For microsatellite loci, we do not favour the hypothesis of null alleles because all F_{IS} estimates were positive, significant and relatively consistent across loci (F_{IS} = 0.165 to 0.330; R_{IS} = 0.072 to 0.0451). It seems highly improbable that all loci exhibit null alleles with such a constant frequency. On the other hand, we cannot reject this hypothesis for the *LDH-C** locus even if selective pressure could also be a possible explanation (Jollivet *et al*, 1995; Allegrucci *et al,* 1997). On the contrary, selection against heterozygotes has never been proven using microsatellite loci which are considered representative of the whole genome.

Assortative mating has been suggested in *Pomatoschistus* species closely related to the sand goby but has never been empirically demonstrated (Lindström, pers.com.). Nevertheless, we believe that the complex mating system of this species involving sexual selection by both sexes is largely responsible for the high F_{IS} values we observed (see Zeh & Zeh, 2001).

Population differentiation over a small geographical scale

In general, our study suggests the existence of two spatially separated breeding units, namely the Oosterschelde estuary and coastal area, while other samples are related to one or the other unit. Comparison of our data with the work of Beheregaray & Sunnucks (2001) and Hoarau *et al.* (2002) leads us to conclude that the structure found in sand goby populations is more complex than the suggested four sub-units detected in the AMOVA analysis. The fixation indices suggest that differences in allele sizes between populations were far more pronounced than differences in allele frequencies (Balloux & Lugon-Moulin, 2002), and especially between the two breeding units (F_{ST} = 0.008 and R_{ST} = 0.022). Nonetheless, gene flow appears to be sufficiently large to swamp any potential for large genetic differences detectable with F_{ST} estimates even if a small differentiation was put forward with our data. Moreover, estimates of gene flow and genetic distances based on F_{ST} values assume that population structure has been stable for sufficiently long to allow an equilibrium

between drift and migration. As a consequence, small F_{ST} values can be observed due to high migration rates in the past, despite little or no current gene flow. Under a strict SMM, R_{ST} will be more sensitive to historical events and to restricted or pronounced gene flow (Balloux & Lugon-Moulin, 2002). The observed R_{ST} value (6%) suggests that the allele shift between the two breeding units is large enough to be explained by isolation of these two populations during the formation of the Holocene coast. Thus, we assume that several processes such as historical events (colonisation of the Oosterschelde by coastal populations) and to a lesser extent restricted actual gene flow due to the geomorphology of the studied area, contributed to the observed differentiation.

Congruence between allozyme and microsatellite loci

The variation of fixation indices among types of markers is one of the most powerful methods for examining whether natural selection has played a role in the observed genetic divergence (see Allendorf & Seeb, 2000). Consequently, discrepancy between allozyme and microsatellite markers has been described in several marine species (Lemaire *et al*, 2000; De Innocentiis *et al*, 2001). It is indicative of either differentiation in one or both markers (Lemaire *et al*, 2000; De Innocentiis *et al*, 2001), to selection or to stochastic events (drift-gene flow) (De Innocentiis *et al*, 2001; McLean & Taylor, 2001). In our data set, microsatellite loci exhibited highest F_{ST} values than allozyme loci, suggesting that the highest mutation rate in microsatellites has increased allele frequency divergence among populations (Allendorf & Seeb, 2000). In addition, none of the alleles scored occurs at high frequency in one population and is absent in the others, as would be observed if new mutations drift to high frequencies within their population of origin before being distributed to others by migration (Allendorf & Seeb, 2000). Thus, our results suggest that the observed differentiation was mainly due to drift and restricted gene flow between the distinct breeding populations (coastal and Oosterschelde groups) and the other samples, but not to any type of selection (see above).

A model for the spatial dynamics of sand goby

Historical and contemporary factors seem to be at play in the spatial dynamics of sand goby in the Southern Bight of the North Sea. Historically, the North Sea coast reached its current shoreline

7,500 years BP as the sea level rose at the end of the last Ice Age (Beets & Van der Spek, 2000). With the rising seawater, gobies must have invaded the area from the English Channel, which can be deduced from the phylogeographical homogeneity of gobies (Paper II) and plaice (Hoarau *et al*, 2002) in the North Sea. As soon as the water was rising, a mesotidal system became functional (van der Molen & de Swart, 2001a). During the Holocene the wind-wave regime changed due to modifications in basin geometry with an increase in wave height (and mixing in the coastal zone) for a constant wave period (van der Molen & de Swart, 2001b). If the West Coast and Oosterschelde estuary represent historical evolutionary units for the sand goby, their separation might be linked to the geography of the estuaries of the Great Rivers (Schelde, Maas and Rhine), which enter the North Sea. For example, the Zeeland coast of the Netherlands only reached most of its current form some 700 years ago. Hence, the question arises whether the North Sea fauna and flora is currently in gene flow – genetic drift – selection - mutation equilibrium.

In a contemporary perspective, sand gobies live in a very dynamic environment with strong tidal currents and experience heavy wave action due to the shallow water depth (Otto *et al*, 1990; Ozer *et al*, 2000). However, their preferred mating habitat seems to focus on two regions in the Southern Bight. Off the west coast of Belgium is a retention zone (Van den Eynde, 1994; Lanckneus *et al*, 2001) where the coastal flow from the English Channel "collides" with the coastal flow from the Rhine/Schelde system. In spring planktonic larval stages of numerous species can be found in this area (Dewicke, 2001). Later on, the postlarvae of many species, including gobies, drift to the nearby inshore nurseries. Off the Voordelta a similar retention zone can be observed, this time driven by a SW coastal current and the NE current originating from the Rhine mouth (Hamerlynck & Mees, 1991). As the genetic structure of sand gobies and plaice (Hoarau *et al*, 2002) reflects historical and contemporaneous factors, it might be termed a divergence-with-gene-flow-system (sensu Beheregaray & Sunnucks, 2001). Unfortunately we do not have any ecological information about the two putative breeding units to assess whether ecological data would be consistent with genetic data and if incipient speciation is occurring. Juveniles of the sand goby are known to undertake migrations from the coastal area and the Oosterschelde to the Westerschelde in fall, presumably to avoid predation and to find food (Maes *et al*, 1998a). This might result in the mixing of genotypes from both breeding units with reduced pair-wise differentiation. We are in the process of clarifying the regional dynamics of the seasonal migration, which are linked to feeding and growth, predation and natural mortality, and habitat selection.

To conclude based on microsatellite loci, our study revealed the existence of two breeding

units presumably connected with a low amount of migrants, consistent with a divergence-with-gene-flow model but not with incipient speciation. This differentiation is most likely attributable to a combination of older historical events, restricted actual gene flow and the complex mating system of sand gobies.

ACKNOWLEDGEMENTS

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CHAPTER V
GENERAL DISCUSSION

In the previous chapters we described the genetic structure and phylogeographic patterns of two sympatric goby species, *Pomatoschistus minutus* and *P. microps* throughout their distributional range (Paper 1, 2 and 3). We have made use of the available knowledge on oceanography and paleoclimatology for interpreting the recurrent genetic structure of either species. We have studied the small-scale genetic structure of *P. minutus* and *P. lozanoi* in the Southern Bight of the North Sea for assessing the influence of environmental variables and local hydrodynamics on regional genetic diversity and differentiation (Paper 4 and 5). In this last chapter we compare (1) the resolution of the various genetic markers which have been employed during this study and (2) the genetic patterns across species of the genus *Pomatoschistus* for assessing the influence of species-specific factors on extant genetic structure.

We have shown a significant differentiation in *P. minutus* and *P. microps* throughout their distributional range and have formulated hypotheses regarding their phylogeographical history. Our data point to a complicated recolonisation history in the northeastern Atlantic Ocean, involving distinct lineages and possibly several waves of colonists. We found evidence for a cryptic species of *Pomatoschistus* sp. in the Northern Adriatic Sea, but results from various markers (Penzo *et al*, 1998; Huyse *et al*, 2002; Paper 2) conflict regarding the timeframe of this speciation event. Speciation processes within the Atlanto-Mediterranean gobies are complex and it is yet unclear which event may have triggered the radiation within the sand goby group (Huyse, 2002).

Hence, a lot of questions remain to be answered. For example, does the peculiar haplotype distribution of *P. microps* around the British Isles result from a range expansion from a glacial refugium in the Southern North Sea or temperature-dependent selection? A more detailed sampling scheme along the eastern English coasts or in the Irish Sea would be required to address this question. We do not know anything about the Baltic *P. microps*, because unfortunately, no samples were available. Similarly, the fine-scale population structure of *P. minutus* and *P. lozanoi* in the Southern Bight of the North Sea remains unclear. We found some evidence for selective pressure acting on a specific locus in *P. lozanoi* in the various habitats which were sampled, but it was not clear which factor might induce selective pressure. Microsatellite analysis of *P. minutus* shows some indications for distinct breeding units but the fine-scale dynamics of *Pomatoschistus* sp. are not known. For example, the spawning and thermal migrations are well described in literature, but

as shown in Paper 4 and 5, sampling on the Belgian Continental Shelf in subsequent years yields completely different results with respect to catches of the various species during the same season. Moreover, the breeding biology of the species, with a complex mating system and high rates of successful sneaking behaviour may contribute to a complex pattern of genetic structure. The unusually high level of variation found in the microsatellites makes interpretation even more difficult.

I. THE CHOICE OF GENETIC MARKERS: AN ASSESSMENT OF THE PERFORMANCE OF ALLOZYMES, MTDNA AND MICROSATELLITES FOR DETECTING GENETIC STRUCTURE IN *POMATOSCHISTUS* SP.

In general allozymes have a lower resolution than DNA for disclosing population genetic structure, in particular when assaying historical processes (Avise, 2000) (but see Daniels *et al*, 2002). Moreover, the problem of the selective neutrality of allozymes continues to influence the interpretation of genetic data (Allegrucci *et al*, 1997; Paper 4). Thus, not unexpectedly, the advent of DNA technology such as PCR and automated sequencers has led to a spectacular increase in DNA-based population genetics studies while allozymes have lost much of their popularity. In this paragraph we compare resolution of the various markers, which were employed throughout the study.

Congruent patterns of large-scale genetic differentiation in Pomatoschistus minutus and P. microps for allozymes and mtDNA

For studying large-scale genetic structure and historical patterns we have employed a combination of a nuclear and a mitochondrial marker. In the case of *P. minutus* we noticed a similar result for allozymes and mtDNA regarding the distinctness of the Adriatic population: both allozymes and mtDNA show that the degree of differentiation between Adriatic and other *P. minutus* is of the same order of magnitude as between *P. minutus* and *P. lozanoi* (Paper 1 and 2). Thus, in the case of the sand goby, both markers have a similar resolution for discriminating cryptic species.

The case of the Adriatic sand goby is but one in a long series of cryptic species, which have been discovered using molecular tools (e.g. Röhner *et al*, 1996; Trewick, 2000; Borsa, 2002; Harris

et al, 2002; Zuccarello *et al*, 2002). Although in many cases a good concordance between molecular and genetic relations between taxa has been found (Wallis & Beardmore, 1984a; Elrod *et al*, 2000; Tinti & Piccinetti, 2000; Young *et al*, 2002), a growing number of studies yields conflicting results between morphology- and genetically-based phylogeny and taxonomy. Fish and invertebrates, for example, are known to exhibit a large degree of phenotypic plasticity, which in the past has led to the description of distinct species, where no genetic differentiation is to be found (e.g. Schwaninger, 1999; Tudela, 1999; Tsigenopoulos *et al*, 2002). Hence, some advocate the use of DNA as a basis for taxonomy instead of the classical morphological criteria (Tautz *et al*, 2003).

At the population level, allozymes don't discriminate as clearly as the mtDNA in this study. Allozymes do not yield any evidence for differentiation between the Western Mediterranean and Atlantic samples (Paper 1), while the mtDNA suggests an historical isolation of the Mediterranean sand gobies followed by secondary contact (Paper 2). Within the Atlantic Ocean both markers show a low degree for population divergence. On the other hand *P. microps* is much more differentiated throughout its range of distribution and this is reflected in both allele frequencies and mtDNA haplotype distribution (Paper 3). In the common goby, allozymes and mtDNA show a remarkable congruence in (1) the structuring of the Atlantic common gobies and (2) the loss of genetic diversity at higher latitudes.

In summary, the overall patterns of large-scale genetic differentiation are similar for both markers in either species: (1) the evidence for allopatric speciation in the Adriatic *P. minutus*, (2) the low degree of within-species differentiation in the Atlantic *P. minutus* and (3) the high degree of population divergence of *P. microps* throughout its whole area of distribution. However, the mtDNA shows a more refined picture of population divergence in both species throughout their distributional range and allows for an historical perspective and the reconstruction of past migration events (paper 2 and 3).

This is congruent with the results of other studies, where similar patterns of population differentiation were found with mtDNA and allozymes (Cook *et al*, 2002; Daniels *et al*, 2002; Tsigenopoulos *et al*, 2002). However, discordance between allozymes and mtDNA has been reported as well, suggesting caution when interpreting genetic data (Turan *et al,* 1998, Piel & Nutt, 2000). Allendorf & Seeb (2000) stressed the importance of examining many loci for assessing patterns of gene flow, while the nature of these makers seemed less important. Especially if a significant level of differentiation is due to the effects of one or few loci, the possibility of selection

acting on a specific locus must always be taken into account, even if so-called 'neutral markers' are employed (Dufresne *et al*, 2002).

Patterns for microsatellites and allozymes in assessing small-scale genetic differentiation: the sand gobies on the sand banks

Nevertheless, allozymes seem to be less suitable for disclosing subtle, small-scale population structure. Allozymes did not show any significant population structuring on the BCS for *P. minutus*, with the exception of differences at one locus, *LDH-C**, which was severely out of Hardy-Weinberg equilibrium. None of the other polymorphic loci showed any small-scale differentiation in allele frequencies. On the other hand, microsatellite analysis yieled some evidence for small-scale population structuring, although patterns remain difficult to interpret. However, cosidernig that no allozyme samples were available from the Oosterschelde, we cannot make any inference about the resolving power for discriminating between Oosterschelde and Belgian coasts.

Recent studies with microsatellites suggest that significant genetic differentiation between geographic populations seems to be the rule rather than the exception in marine organisms, even in species with long-lasting pelagic larval stages, capable of extensive migrations (Nesbø *et al*, 1999; Shaw *et al*, 1999; Wirth & Bernatchez, 2001). However, Hansen *et al* (2001) warn against the pitfalls of misscoring microsatellite alleles and underlines that any significant ´subtle´ genetic structure should be backed up by other relevant information. For example, genetic differentiation in highly vagile species such as Atlantic cod (Ruzzante *et al*, 1998) and sea bass (Bahri-Sfar *et al*, 2000) is consistent with oceanographic features in the area of distribution. In case of the sand goby, the suggestion of separate breeding units in the Oosterschelde and the Belgian coast is supported by sampling surveys showing aggregation of larvae in distinct areas off the Belgian coast (Dewicke, 2001), while the Oosterschelde is considered a self-sustaining ecosystem (Scholten *et al*, 1990). The evidence for 'Selective Tidal Stream Transport' in *Pomatoschistus* gobies (Bardin & Pont, 2002) shows that gobies are capable of active migratory behaviour during the larval stage. Thus, both behavioural and life history characteristics of the species and the hydrology of the area provide support for more complex processes than simply larval drift via oceanic currents.

As a final remark, we conclude that any genetic marker is valuable, provided (1) the right questions are addressed, and (2) results are interpreted with caution. In this study we have shown

that allozymes have a similar resolution as mtDNA for detecting cryptic species (Paper 1 and 2) and global population differentiation. In this case the use of allozymes might be the preferred tool over more sophisticated, expensive and time-consuming DNA methods. At the other hand, their resolution for detecting small-scale or recent evolutionary processes is not sufficient (Paper 5) and the possibility of selection acting on certain loci should always be taken into account (Paper 4; Lemaire *et al*, 2000). Microsatellites may be useful for studying small-scale evolutionary processes, but their high variability and consistent heterozygote deficits may be a problem for interpretation.

II. COMPARING LARGE-SCALE PATTERNS OF GENETIC STRUCTURE AND PHYLOGEOGRAPHY IN *POMATOSCHISTUS MINUTUS* AND *P. MICROPS*

We have shown that oceanographic and paleoclimatological features play a major role in shaping population structure in *Pomatoschistus minutus* and *P. microps* (Paper 2 and 3). The overall genetic differentiation within each species is consistent with larval dispersal via oceanic currents in the northeastern Atlantic Ocean, while at least a partial barrier for dispersal exists between Atlantic and Mediterranean waters.

However, other important agents, which may influence population structure, are species-specific features such as behaviour, ecology and life history characteristics (Wilke & Davis, 2000; Beheregaray & Sunnucks, 2001; Dawson *et al*, 2002). A stenotopic species may find itself 'trapped' in its habitat due to ecological constraints despite a possibly high potential for dispersal. For example, a lowered level of salinity at the mouth of a river may represent a barrier to dispersal for marine organisms (Rocha *et al*, 2002). Reproductive isolation can be enhanced in populations evolving under different ecological conditions (Rundle *et al*, 2000). As pointed out by Bernatchez & Wilson (1998), co-occurring species with distinct ecological characteristics may respond differently to paleoclimatological events and thus show different phylogeographic patterns across the same range. Consequently, sympatric sister species with a similar mode of reproduction but different ecological requirements provide an excellent opportunity for assessing the influence of ecological constraints on their extant genetic structure (Rocha *et al*, 2002).

P. minutus and *P. microps* occur sympatrically over a large part of their distributional range and show a similar mode of reproduction with a pelagic larval stage lasting from one to two months. In the Northeastern Atlantic both species spawn between April and August (Jones & Miller, 1966;

Fonds, 1973). Mediterranean *P. microps* show a protracted breeding season from March to September, while *P. minutus* reproduces during winter from December to April (Bouchereau & Guelorget, 1997). When considering the spawning site, Fonds (1973) and Claridge *et al* (1985) suggested a spawning migration towards the sea of estuarine sand gobies. Very low numbers of both *P. minutus* and *P. microps* in estuaries during the breeding season, but high numbers in fall and winter substantiate this (Maes *et al*, 1996; Laffaille *et al*, 2000). However, both *P. minutus* and *P. microps* are able to reproduce in the estuarine environment (Vaas, 1970; Arruda *et al*, 1993). Thus, although both species are able to spawn in a brackish-water environment, reproduction takes essentially place at sea, at least in the Atlantic Ocean. *P. microps* breeds in very shallow areas up to 1 m depth while *P. minutus* reproduces in slightly deeper water between 10 and 25 m (Fonds, 1973). Contrary to Atlantic common gobies, Mediterranean *P. microps* spend their whole lifecycle sedentary in estuarine and lagoon-like environments (Pampoulie, 2001), while Mediterranean *P. minutus* undertake seaward spawning migrations (Pampoulie *et al*, 1999).

A substantial number of studies have dealt with comparative analyses of phylogeography and population genetic structure across (more or less related) marine species. However, most focus on species with contrasting life histories and dispersal capacities (e.g. Bowen & Avise, 1990; Riginos & Victor, 2001). The general pattern which emerges is that organisms with direct development or a short pelagic larval stage are more genetically differentiated than those with a longer lasting pelagic larval stage (Doherty *et al*, 1995; Goldson *et al*, 2001; Rocha *et al*, 2002). Species requiring an estuarine environment for reproduction are usually also more differentiated than those spawning at sea, due to the constraints imposed by their habitat requirements (Dawson *et al*, 2002).

We address here two phylogenetically and ecologically similar species for assessing the influence of species-specific factors in shaping population structure. More specifically, we test the following hypotheses:

(1) As described above, Atlantic populations of both species have a similar potential for dispersal with an extended pelagic larval stage, while adult fish are adapted to a demersal life-style. Thus, if the population structure in *P. minutus* and *P. microps* is the result of an exclusively passive larval dispersal controlled by oceanic currents, we might expect a similar degree of genetic differentiation within both species. If this is not the case, then behavioural and/or ecological factors may have to be invoked for explaining differences.

(2) In the Mediterranean Sea, estuarine *P. microps* are living sedentarily in semi-enclosed lagoons during their whole life cycle, while *P. minutus* reproduces at sea. Thus, we may expect a higher level of differentiation for *P. microps* than for *P. minutus* between the Atlantic Ocean and the Mediterranean Sea.

Species-specific patterns of genetic differentiation and phylogeography in *Pomatoschistus minutus* and *P. microps*

We have shown that the patterns of genetic differentiation as detected with mtDNA look similar for *Pomatoschistus microps* and *P. minutus* (Papers 2 and 3). In both species we have recorded (1) that the highest amount of genetic differentiation is due to differences between the samples from the Atlantic Ocean and the Mediterranean Sea and (2) a pattern of isolation-by-distance. Likewise, overall phylogeographic patterns in *P. microps* and *P. minutus* seem similar. A shallow phylogeographic structure with a high haplotype diversity and a low nucleotide diversity is not uncommon among marine fish, and is considered as the signature of either a bottleneck or a founder event and subsequent population expansion (Grant & Bowen, 1998). Both species show (1) a star-like phylogeny with a few common (ancestral) haplotypes and a large number of rare radiating variants and (2) a negative correlation between haplotype diversity and latitude, at least in the Atlantic basin. However, a closer look at the data shows some striking differences in genetic patterns between these sympatric goby species.

(i) Pomatoschistus sp. have different patterns of genetic differentiation across the Atlanto-Mediterranean boundary

Although both *Pomatoschistus. minutus* and *P. microps* show a genetic break across the Strait of Gibraltar, the magnitude of genetic differentiation between the Atlantic and the Mediterranean *P. microps* is definitely higher than for *P. minutus:* allozymes showed no divergence between *P. minutus* from the southern North Sea and the western Mediterranean (Paper 1), while significant differences in allele frequencies are recorded between *P. microps* from the western Mediterranean and the geographically much closer sites of Faro (southern Portugal) and La Tremblade (Gulf of

Biscay) (Paper 3). Pair-wise values of F_{ST} and genetic distance between Atlantic and Mediterranean samples are higher for *P. microps* than *P. minutus* (Table 5.1 and 5.2). The same haplotype in *P. minutus* (A) is recorded in high frequencies across the whole range of the species, including the western Mediterranean Sea. On the contrary, the only haplotype in *P. microps* shared between the Atlantic Ocean and the Mediterranean Sea is the haplotype present in the Norwegian Sea, at the northernmost edge of the species' distribution.

Table 5.1: Allozyme-based pair-wise F_{ST} values (Weir & Cockerham, 1984) of *Pomatoschistus minutus* (upper triangle) and *P. microps* (lower triangle) based on allozyme electrophoresis as compiled from Papers 1 and 3.

	Doel	Texel	Oban	Pérols
Doel	0.000	0.035	0.121	-0.004
Texel	0.065	0.000	0.306	0.035
Oban	0.310	0.346	0.000	0.141
Pérols	0.290	0.282	0.308	0.000

Table 5.2: mtDNA-based pair-wise genetic distances (Tamura-Nei, 1993) of *Pomatoschistus minutus* (upper triangle) and *P. microps* (lower triangle) as compiled from Papers 2 and 3.

	Trondheim	Oban	Texel	Doel	Plymouth	Pérols
Trondheim	0.000	0.027	0.071	0.337	0.008	0.234
Oban	0.955	0.000	0.133	0.455	0.044	0.342
Texel	0.862	0.009	0.000	0.078	0.026	0.243
Doel	0.642	0.158	0.128	0.000	0.257	0.338
Plymouth	0.690	0.553	0.544	0.229	0.000	0.229
Pérols	0.336	0.771	0.738	0.518	0.574	0.000

(ii) The higher population differentiation of P. minutus compared to P. microps within the Atlantic Ocean

Within the Atlantic basin, *P. minutus* is more differentiated than *P. microps*. In *P. minutus*, the occurrence of haplotype A at all sampling sites, ranging from the Norwegian Sea and the Baltic Sea south to the western Mediterranean in appreciable frequencies, points to an extensive amount of gene flow throughout its distributional range. On the contrary the Atlantic haplotypes of *P. microps* show a much more restricted range of distribution and no haplotypes common to all populations are recorded. Haplotype H4 has a clear boreal distribution (north of the English Channel), while the H1 mainly recorded distribution south of the English Channel. *P. microps* from the English Channel and the Gulf of Biscay are clearly differentiated from the western Scottisch and Irish coast, while the *P. minutus* from these sites are grouped together (Fig.5.1 and 5.2).

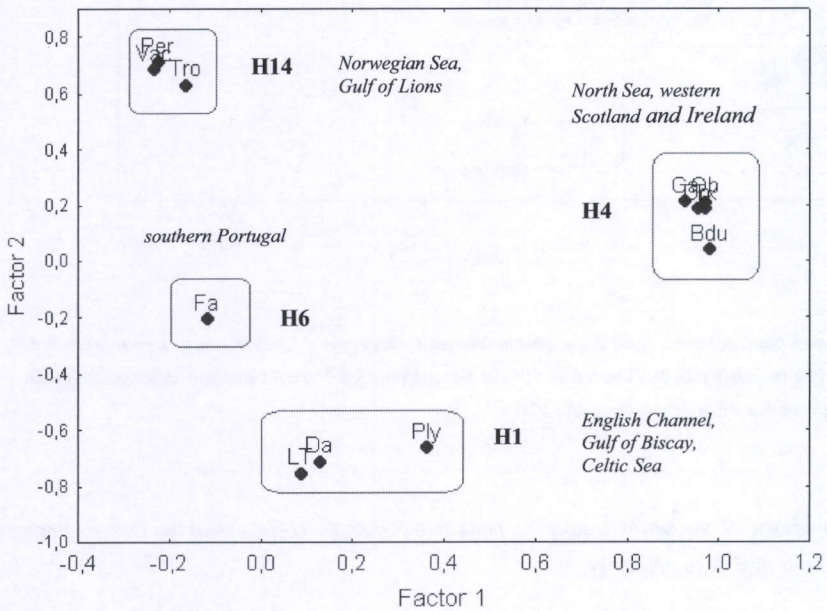

Fig. 5.1: *Pomatoschistus microps*: Principal Component Analysis based on haplotype frequencies. Singletons are excluded. Factor 1 and 2 explain 40.96 % and 25.41% resp. of the total variance. The most common haplotype of each group is indicated. For sample abbreviations, see Table 3.1.

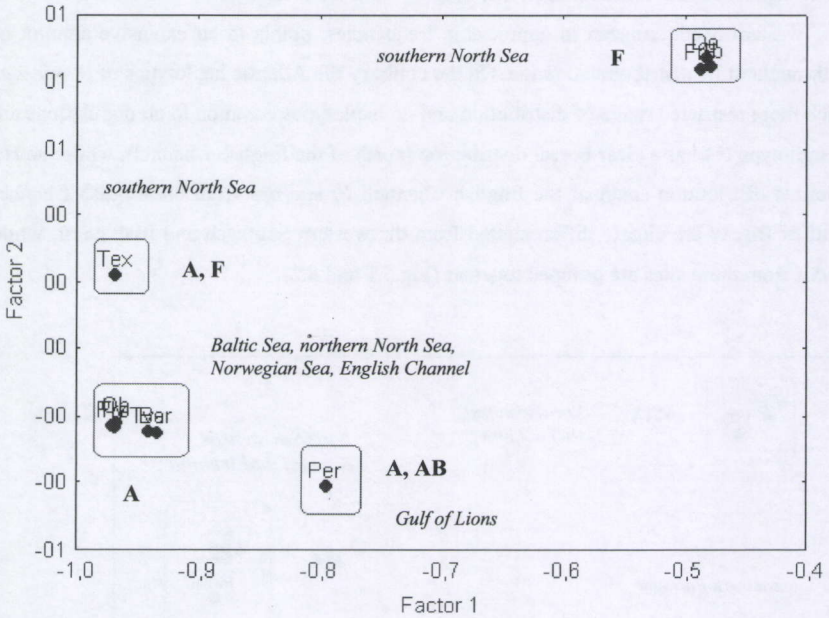

Fig. 5.2: *Pomatoschistus minutus.* Principal Component Analysis on haplotype frequencies. Singletons are excluded. The first two factors explain resp 70.94 % and 24.51 % of the total variance. The most common haplotype(s) of each group are indicated. For abbreviations see Table 2.2.1.

(iii) A comparison of the phylogeographic patterns of Pomatoschistus minutus and P. microps throughout their distributional range

The phylogeographic patterns of both species point to a postglacial range expansion in the Atlantic with a possible glacial refugium along the southern British coast or in the Southern Bight of the North Sea (Paper 2 and 3). The presence of haplotype A in *P. minutus* at all sampling sites in

appreciable frequencies suggests lineage sorting of this haplotype followed by extensive gene flow throughout the area of distribution. The distinct western Mediterranan haplotypes which form a separate cluster are presumable older ones, while the presence of haplotype A in this area is explained as secondary contact after range expansion from the Atlantic into the Mediterranean Sea. The haplotype distribution of the common goby, on the other hand, requires a more complicated explanation. The occurrence of H14 at the edges of the species range suggests that this haplotype might have a more ancient distribution and has been displaced by H1 and H4 in a later stage of the recolonisation process. The peculiar distribution of H1 versus H4 around the British Isles might be attributed to several reasons, such as a postglacial range expansion from a refugium in the Southern Bight or temperature dependent selection. The distinct phylogeographical patterns within either species are summarised in Fig. 5.3 and 5.4 and a hypothetical scenario for the migration routes of the various haplotypes in either species is presented.

A scenario for the postglacial recolonisation of the Atlantic coasts by Pomatoschistus microps

We suggest that a population of H14 -*P. microps* invaded the Atlantic Ocean from an ancestral Mediterranean population and gave rise to the Atlantic haplotypes of *P. microps* along the Iberian coast. *P. microps* expanded its range northward along the European coasts. At the onset of the Weichselian, *P. microps'* range was severely contracted and the species survived in a refugium along the Portuguese coast. Nevertheless, a H4-population may have survived in the glacial lake in the Southern Bight. Upon deglaciation, H14 *P. microps* migrated northward and recolonised the coasts of western Europe (Fig. 5.3a). In the Southern Bight of the North Sea, H14 might have come into contact with H4, which was also expanding its range northward into the North Sea. H4 displaced H14 in the North Sea. H14 became rare and only survived in large numbers in the northern part of the distributional area.

Subsequently, haplotype H1 migrated northward from its southern refugium and displaced H14 along the Atlantic coasts south of the English Channel. Areas more to the north were occupied by H4 and H1 did not manage to intrude (Fig. 5.3b).

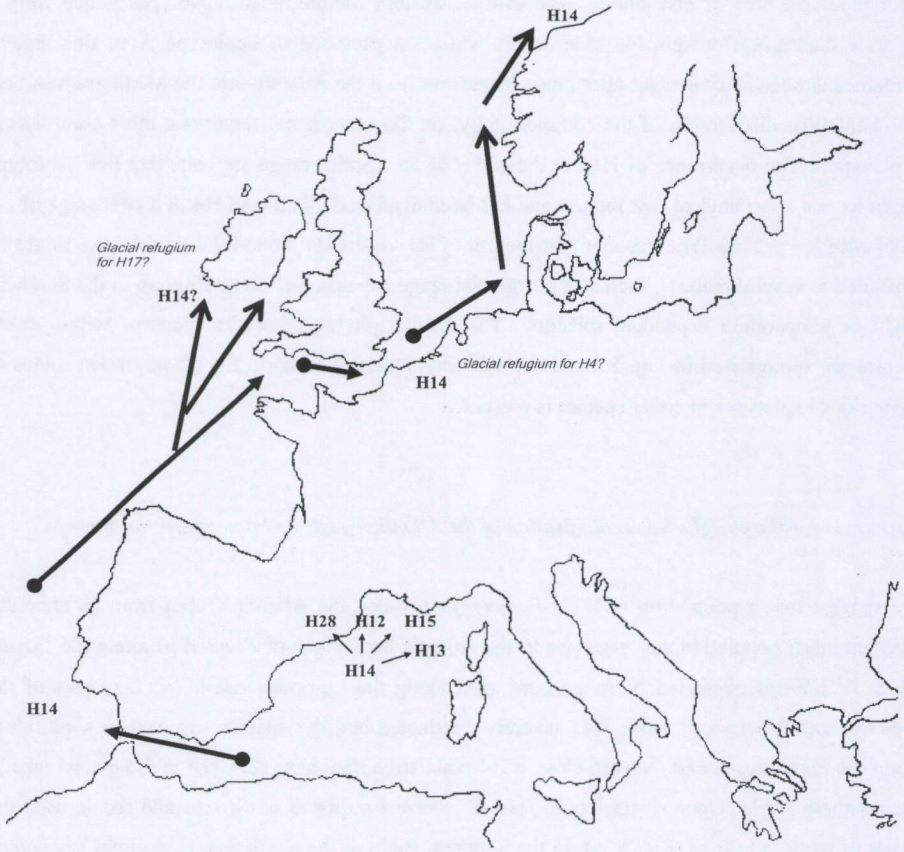

Figure 5.3a). *Pomatoschistus microps* : Hypothetical migration routes. A first wave of colonisation: the northeastern Atlantic Ocean by H14.

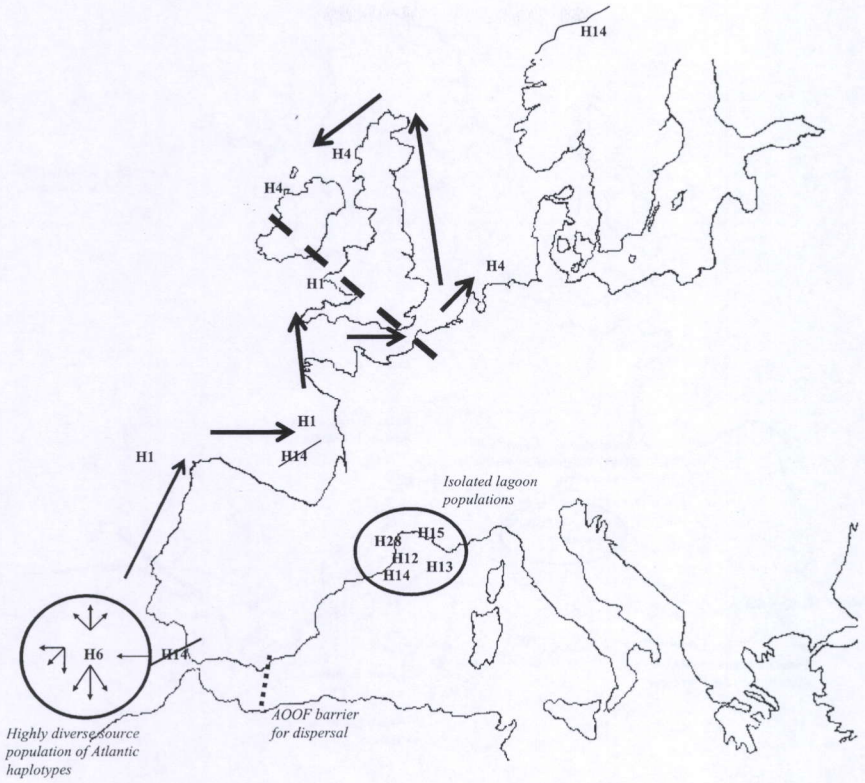

Figure 5.3b). *Pomatoschistus microps* : Hypothetical migration routes. Displacement of H14 in the Atlantic basin by H1, H4 and decendants. AOOF: Almería-Oran Oceanic Front

Pomatoschistus minutus: proposed recolonisation routes

A population of a proto- *P. minutus* ancestor gave rise to the three species in the *P. minutus* complex and the Adriatic sand gobies in the Mediterranean Sea. These colonised lagoons and estuaries in the Mediterranean Sea. A population invaded the Atlantic Ocean, giving rise to haploytpe A and its descendants (Fig. 5.4a).

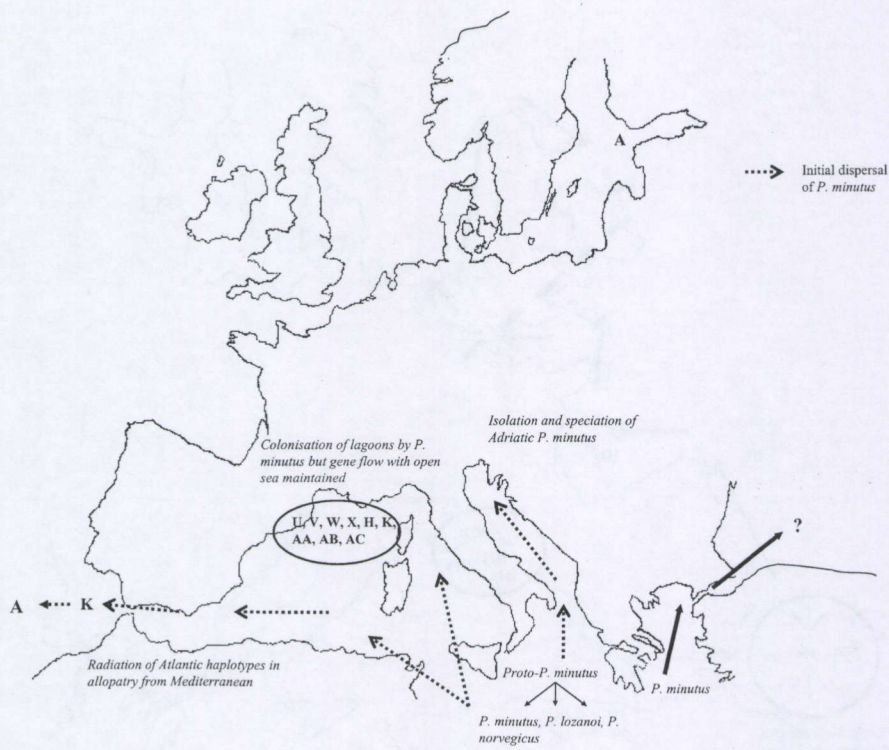

Figure 5.4a). *Pomatoschistus minutus*: Hypothetical migration patterns: Origin of *P. minutus* complex in the Mediterranean Sea, isolation in the Adriatic Sea, colonisation of Western Mediterranean lagoons and dispersal in the Atlantic.

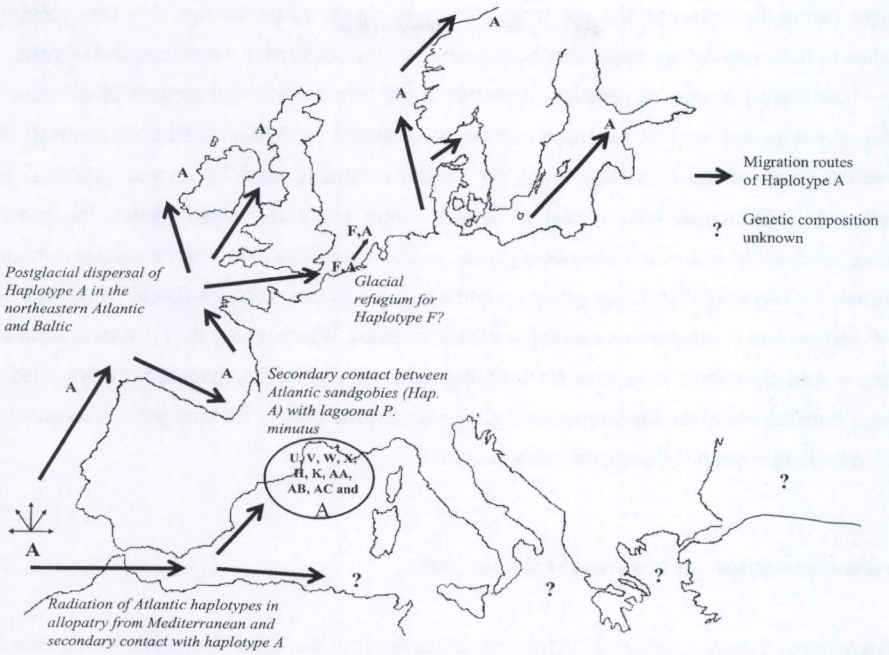

Figure 5.4b). *Pomatoschistus minutus*: Hypothetical migration patterns. Origin of Atlantic haplotypes and secondary contact with Mediterranean sand gobies.

Differentiation of *P. minutus* and *P. microps* within the Mediterranean Sea

Because the Rhône Delta, where both sampling sites are located, finds its origin in the late Pleistocene and postglacial sea-level rise, dating back only 18 ka BP (Arnaud-Fasset, 2002), colonisation of the various lagoons belonging to the system by *P. minutus* and *P. microps* cannot be of an older date. At the moment, no direct connection exists between Vaccarès and Pérols (60 km apart) except via the sea (Pampoulie, pers. comm). No differentiation was found between the *P. microps* from Vaccarès and Pérols, implying recent isolation, or else genetic drift would have resulted in differences between the sites. Historically, both sites were connected and became separated during the Holocene development of the Rhône Delta, which underwent several major

changes during the Holocene, the last of which was during the Little Ice Age (17- 18th century). Isolation of these populations might thus be as recent as a few hundred to a few thousand of years.

The limited amount of genetic differentiation between Atlantic and western Mediterranean *P. minutus* does not warrant subspecies status as proposed by Miller (1986). In contrast, the taxonomic status of the *P. minutus* from the northern Adriatic must be revised. Although no morphometric differences with typical *P. minutus* were found (Stefanni, 2000a), the genetic distance as observed with two independent genetic markers is sufficient to warrant species status, as confirmed by Huyse (2002). If one prefers not to designate species status, it should be pointed out that *P. lozanoi* and *P. minutus* have a similar genetic distance. Whether only the *P. minutus* from the Northern Adriatic consist of a separate breeding unit, or whether the complete Adriatic Sea is isolated from the rest of the Mediterranean with respect to gene flow in the sand goby, remains to be verified by further sampling along the Adriatic coast.

Are there species-specific behavioural mechanisms?

Pomatoschistus minutus realises a higher rate of dispersal at the same geographic scale than *P. microps* despite a similar potential and a similar exposure to hydrographical patterns. Rocha *et al* (2002) showed that adult habitat requirements play a major role in population divergence. However, *P. minutus* and *P. microps* occur sympatrically over a large part of their distribution, and the fish examined in this study were caught in the same sample. Similarly, although both species must have been subjected to the same paleoclimatological and vicariance events, phylogeographic patterns are different (Fig 5.3 and 5.4) as well. Secondary contact between Atlantic and Mediterranean sand gobies is suggested while the Mediterranean common gobies seem to completely isolated in the sites sampled. Thus, the question arises how these different patterns in sympatric species with a smilar potential for dispersal might be reconciled.

Clearly, although topographical, oceanographical and paleoclimatological features represent the framework for the interpretation of the genetic structure of marine species, this may not be sufficient. For example, Bahri-Sfar *et al* (2000) suggested that, besides purely hydrographical factors, behavioural traits may be partly responsible for the observed genetic differentiation between Atlantic, Western Mediterranean and Eastern Mediterranean sea bass. Among behavioural traits

enhancing population differentiation described in literature are (1) **active homing behaviour** and (2) **selective tidal stream transport** (Greer Walker *et al*, 1978).

Homing behaviour implies fidelity to distinct, natal spawning sites, and is well-documented in anadromous species such as salmonids (Laikre *et al*, 2002) and sturgeon (Waldman *et al*, 2002), migrating upstream in their natal rivers for spawning. The behaviour has been reported in a number of marine fish, such as cod (Green & Wroblewski, 2000), herring (Sinclair, 1988) and weakfish (Thorrold *et al*, 2001). However, as pointed out by McQuinn (1997), a prerequisite for genetic isolation is not just homing behaviour, but *natal homing*. Herring, for example, shows a strong homing behaviour, but not necessarily to natal spawning sites. Adults are fidel to spawning areas but these are often not their natal spawning areas. In fact, this straying behaviour would lead to a considerable amount of gene flow between populations (Smith & Jamieson, 1986).

Pampoulie *et al* (Paper 5) have shown that high resolution markers yield evidence for distinct breeding units of the sand goby in the Southern Bight, with one putative spawning area off the Belgian west coast, while another may be situated off the Voordelta along the southwestern coast of The Netherlands. However, even if local populations of *Pomatoschistus* spp. breed at distinct sites along the coast, the pelagic larvae have plenty of opportunities to disperse over large distances on the currents in the area.

Selective tidal stream transport (STST) has been reported in larvae and adults of a large number of fish and invertebrates (e.g. McCleave & Kleckner, 1982; Metcalfe *et al*, 1990; Arnold *et al*, 1994). Basically, a net horizontal transport is achieved by vertical movements of the organisms in and out of the water column, which are synchronised with the direction of the tidal currents. STST can be used for north-south migrations along the coast or movements into estuaries, or else as a retention mechanism in other species (Forward & Tankersley, 2001). Considering the large variety of species in which the phenomenon has been described, STST seems to be the rule rather than the exception in the movements of marine organisms (for a review, see Forward & Tankersley, 2001) and is especially important for demersal species with limited swimming abilities. Bardin & Pont (2002) reported STST for both juvenile and adult *Pomatoschistus* spp. when entering a Mediterranean lagoon.

The Atlanto-Mediterranean boundary: to cross or not to cross

The Almería-Oran Oceanographic Front (AOOF), situated east of Gibraltar in the Alboran Sea, is the true boundary between Atlantic and Mediterranean water masses (Tintore *et al*, 1988). Oceanic fronts are known to act as barriers for the dispersal of marine pelagic larvae (Goldson *et al*, 2001). However, various marine species with a long-lived planktonic larval stage respond differently to the presence of the Almería-Oran front. For example, no genetic differentiation was found between Atlantic and western Mediterranean samples of European anchovy (Magoulas *et al*, 1996; Borsa, 2002) and swordfish (Pujolar *et al*, 2002), reflecting a significant amount of ongoing gene flow between both basins. At the other hand, significant genetic differences east and west of the Almería-Oran front were found for e.g. sea bass (Allegrucci *et al*, 1997), flounder (Borsa *et al*, 1997b), hake (Roldán *et al*, 1998), mussels (Quesada *et al*, 1995) and krill (Zane *et al*, 2000). Clearly, species-specific factors other than purely passive larval dispersal must play a role in maintaining either genetic homogeneity or reproductive isolation between Atlantic and Mediterranean populations of marine organisms. If *Pomatoschistus minutus* and *P. microps* were retained passively in the gyres east or west of the AOOF, then a similar degree of population differentiation should be reported in both species, which is obviously not the case. Two non-mutually exclusive hypotheses are suggested for explaining this discrepancy:

(1) That the level of genetic divergence is higher between Mediterranean and Atlantic *P. microps* than in *P. minutus,* may be explained by their different life histories. *P. microps* inhabiting the Mediterranean lagoons spend their whole life cycle, including reproduction, in semi-enclosed lagoonal and estuarine environments, while Mediterranean *P. minutus* spawn at sea. In the Alboran Sea, a net inflow of less dense Atlantic water at the surface is complemented by an outflow of more saline Mediterranean water at depth (Krijgsman, 2002). Thus, any passive gene flow of pelagic *Pomatoschistus* sp. larvae between the two basins is probably unidirectional from the Atlantic Ocean into the Mediterranean Sea. Even if pelagic larvae manage to migrate into the western Mediterranean Sea, the probability of Atlantic *P. microps* mixing with Mediterranean conspecifics must be small, considering the geographic isolation of the latter. In that respect, *P. microps* larvae drifting across the AOOF may respond to what is described as 'vagrants' in the member-vagrant hypothesis of Sinclair (1988). 'Vagrants' have a higher dispersal rate than 'members' but at the same time risk not to find a suitable habitat or partner and thus may represent a loss to the gene pool. Possibly, these ecological differences between Mediterranean *P. microps* and *P. minutus*

could also explain the strikingly low level of genetic variation in the Mediterranean lagoons for *P. microps* compared to *P. minutus*: if the ancestral population of common gobies invading the lagoon was small or genetically homogeneous, the recurrent isolation of common goby populations in the lagoons will prevent gene flow and increase of genetic variation. Because Atlantic and Mediterranean *P. minutus* reproduce at sea and hence have marine larvae, Atlantic sand goby larvae drifting into the Mediterranean could mix with these Mediterranean sand gobies and move with them into the estuaries.

(2) *P. minutus* and *P. microps* may show species-specific behavioural differences with respect to active migration. As *P. minutus* and *P. microps* are able to spawn both in the estuarine and the marine environment, larvae must physiologically be able to support large fluctuations in salinity as encountered across the AOOF. Adult *Pomatoschistus* spp. are considered poor swimmers but, considering the spawning and thermal migrations, they are able to cover substantial distances. Thus, there is no *a priori* reason to assume that *P. minutus* and *P. microps* would not be physically able to cross the AOOF. Naciri *et al* (1999) stressed that a purely passive larval retention on either side of the AOOF may not be sufficient for explaining the observed differentiation in euryhaline species with a long-lived planktonic stage. It was proposed that non-passive mechanisms such as homing behaviour of spawning fish or selection against larvae that cross the front may play a role in maintaining genetic isolation. Adults of the common goby are clearly better adapted to a coastal-estuarine habitat than a fully marine habitat, because they are rarely recorded off-shore (Miller, 1986; own obs.). Beyst *et al* (2001) reported that adult *P. microps* reside only temporally in the surf zone along the Belgian coast, mainly during the reproductive season, and found no juvenile *P. microps* in the area. Similarly, Dewicke (pers. com) recorded mainly juvenile *P. minutus* and *P. lozanoi* along the Belgian coast. Thus, it seems that *P. microps* larvae migrate into estuaries, while young *P. minutus* use both estuaries and surf zones for development. *P. microps* larvae which drift into estuaries using STST may be selected for, which would enhance population differentiation. Adult *P. minutus* at the other hand, are well-adapted to both estuarine and fully marine conditions. Hence, in the latter species there might be less selective pressure on larvae to migrate into the estuarine environments, providing them with more opportunities for long-distance dispersal.

Conclusive remarks

This final chapter highlights the importance of comparing ecologically distinct species when interpreting patterns of population differentiation and phylogeography throughout an geographical area. It has been shown that sympatric species with a similar dispersal potential may respond differently to hydrological and paleoclimatological events, suggesting that active migratory or non-migratory behaviour may be more important in shaping population structure than usually accounted for.

SUMMARY

Large-scale and small-scale genetic patterns in three species of marine gobies, *Pomatoschistus minutus* (Pallas, 1770), *P. lozanoi* (de Buen, 1923) and *P. microps* (Krøyer, 1838) were studied. We used the available knowledge of the biology of the species and past and present-day oceanographic features throughout their distributional range for inferring population genetic structure and phylogeographical history of small demersal fish species along the European coasts. These three species occur sympatrically throughout a large part of their distributional range, which comprises the European Atlantic coasts and the Mediterranean Sea. They have a similar potential for dispersal with an extended pelagic larval stage, but differ ecologically.

Small-scale patterns along the Belgian coast were studied with allozyme electrophoresis at fourteen putative loci. No evidence for population differentiation was found, except for a clinal change in allele frequencies at one locus in *P. lozanoi*. We suggest that this is due to differential selective pressure in distinct habitats (resp. estuarine, coastal and marine), rather than reproductive isolation. Contrary to other studies, we did not find any correlation between environmental heterogeneity and levels of genetic diversity.

For studying large-scale genetic structure we employed a combination of allozyme electrophoresis, SSCP- and sequence analysis of fragment of the cytochrome b locus on the mtDNA. Overall patterns of *P. minutus* and *P. microps* revealed (1) the highest amount of divergence between Atlantic and Mediterranean species, (2) a pattern of isolation-by-distance, consistent with a larval dispersal via oceanic currents and (3) a shallow phylogeographical structure with a few common (ancestral) haplotypes and a large number of rare variants radiating. In the Atlantic basin, phylogeographical analysis of both species points to a range expansion into northern areas with a loss of variation at higher latitudes and a possible glacial refugium in the English Channel or the Southern Bight. However, despite similar overall patterns, striking interspecific differences were recorded. *P. microps* is much more differentiated than *P. minutus* on the same scale, and has a different phylogeographical history in the Mediterranean Sea. We suggest that this is due to ecological constraints on dispersal in the estuarine-marine *P. microps*. We postulate that species-specific behavioural patterns play a more important role in shaping population structure than usually accounted for.

SAMENVATTING

In deze studie hebben we de genetische structuur van drie soorten mariene grondels, het dikkopje, *Pomatoschistus minutus* (Pallas, 1770), P. lozanoi (de Buen, 1923) en *P. microps* (Krøyer, 1838) onderzocht. We hebben gebruik gemaakt van de beschikbare informatie over (1) de biologie van de soorten en (2) oceanografische patronen en paleoclimatologie voor de interpretatie van de populatiegenetische structuur en fylogeografie van kleine demersale vissen langs de Europese kusten. Deze drie soorten komen sympatrisch voor over een groot gedeelte van hun verspreidingsgebied, dat de Europese Atlantische kusten en de Middellandse Zee omvat. Ze hebben een gelijkaardige dispersiecapaciteit met een pelagisch larvaal stadium gedurende een aantal weken, maar vertonen interspecifieke ecologische verschillen.

Kleinschalige genetische structuur werd onderzocht met allozyeme-electroforese voor *P. minutus* en *P. lozanoi* op het Belgisch Continentaal Plat en het Schelde-estuarium. Allelefrequenties van veertien loci werden bestudeerd. Er werden geen significante verschillen gevonden tussen grondels afkomstig van verschillende staalnamesites, met uitzondering van een clinale verandering in de allelfrequenties op één locus in *P. lozanoi*. We suggereren dat deze verschillen het gevolg zijn van differentiële selectiedruk in de verschillende habitats (estuarien, kust en marien) en neit van reproductieve isolatie. In tegenstelling tot andere studies vonden we geen verband tussen niveau van genetische diversiteit en graad van heterogeneïteit in de verschillende habitats.

Grootschalige genetische structuur werd onderzocht met behulp van allozymelektroforese, en SSCP-en sequentieanalyse van een fragment van het cytochroom b locus op het mtDNA. Globale analyse van de genetische structuur op de schaal van het verspreidingsgebied toonde in beide soorten gelijkaardige patronen: (1) de grootste mate van differentiatie is te wijten aan de verschillen tussen het Atlantische en Middellandse Zee bekken, (2) een patroon van isolatie-door-afstand, consistent met een verspreiding van de larven via oceanische stromingen en (3) een ondiepe fylogeographische structuur met enkele algemene haplotypes en een groot aantal zeldzame. In het Atlantisch bekken werden aanwijzingen gevonden voor een noordelijke expansie met een verlies aan diversiteit op hogere breedtegraad en een mogelijk glaciaal refugium in de Zuidelijke Noordzee of het Engels Kanaal voor beide soorten.

Ondanks de globale gelijkenissen vertoont *P. microps* een hogere graad van differentiatie op eenzelfde schaal dan *P. minutus* en een verschillend fylogeografisch patroon in de Middellandse

Zee. We suggereren dat dit gevolg is van ecologische beperkingen van dispersie in deze estuariene-mariene soort.

We stellen bijgevolg dat soortspecifieke gedragingen een belangrijker rol spelen bij het ontstaan van populatiestructuur dan doorgaans wordt aangenomen.

SAMMENDRAG

Denne avhandling omhandler undersøkelser over genetiske struktur i tre arter av marine kutlinger, sandkutlingen *Pomatoschistus minutus* (Pallas, 1770), lozano kutlingen *P. lozanoi* (de Buen, 1923) og leirkutlingen, *P. microps* (Krøyer, 1838). Vi har brukt dataene om (1) artenes biologi og (2) oseanografiske mønstrer og paleoklimatologi for å tolke populasjonsgenetiske strukturen og fylogeografien til små bunnfisk langs europeiske kystene. Vi har brukt en kombinasjon av allozymelektroforese, SSCP- og sekvensanalyse av mitochondrial DNA.

Disse tre artene forekommer sympatrisk i store deler av utbreddelsesområdet. Dette omfatter de europeiske Atlanterhavskystene og Middelhavet. Artene har det samme potensiale for spredning i og med at de har et pelagisk larvestadium som varer i flere uker, men viser artsspesifikke økologiske forskjeller.

Småskale genetisk differensiering er blitt undersøkt langs kysten av Belgia basert på allozymelektroforese. Vi fant ingen tegn til signifikante forskjeller mellom prøver fra ulike innsamlingslokaliteter med unntak av en gradvis variasjon i allelfrekvenser på et locus i *P. lozanoi*. Vi antar at årsaken til dette heller er ulikt seleksjonspress i de forskjellige habitater, enn reproduktiv isolasjon. I motsetning til andre studier fant vi fann ingen forbindelse mellom nivået av genetisk variasjon og graden av heterogenitet for de ulike habitatene.

Storskale genetiske strukturmønstre ble funnet stort sett å være lik for sandkutlingen og leirkutlingen, og det viser (1) største grad av differensiering er mellom Atlantiske og Middelhavspopulasjoner av begge artene, (2) isolasjon-over-avstand, som er konsistent med vandring til larvene på grunn av oseaniske strømmer og (3) en enkel fylogeografisk struktur med få vanlige og mange sjeldne haplotyper. I Atlanterhavet, fylogeografiske analyser viser en nordlig utbreddelse med tap av genetisk variasjon i de høyere breddegrader, og muligens en glasial refuge i Den engelske kanalen eller sørlige Nordsjøen.

Til tross for likheten fann vi iøynefallende artsspesifikke forskjeller. Leirkutlingen har en mye høyere grad av populasjonsdifferensiering enn sandkutling over samme geografiske skala, og de viser ulike fylogeografiske mønstre Middelhavet. Vi antar at artsspesifikk adferd er viktigere enn det som vanligvis blir antatt ved dannelsen av populasjonsstruktur.

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